University of Alberta

Library Release Form

Name of Author: Maximillian Rosario

Title of Thesis: Studies on the isolation of an antibacterial peptide produced by *Carnobacterium piscicola* UAL26.

Degree: Master of Science

Year this degree granted: 2001

University of Alberta

Studies on the isolation of an antibacterial peptide produced by *Carnobacterium piscicola* UAL26.

by

Maximillian Rosario

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Food Science and Technology

Department of Agricultural Food and Nutritional Science

Edmonton, Alberta

Fall, 2001

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Studies on the isolation of an antibacterial peptide produced by *Carnobacterium piscicola* UAL26 submitted by Maximillian Rosario in partial fulfillment of the requirements for the degree of Master of Science in Food Science and Technology.

Dedication

This thesis is dedicated to Dr. J.A. Rosario.

Abstract

Carnobacterium piscicola UAL26 is a lactic acid bacterium that was isolated from vacuum packaged ground beef. The organism produces a proteinaceous antibacterial compound capable of inhibiting a broad spectrum of gram-positive bacteria, including pathogenic bacteria. A peptide similar to β-casein was isolated from culture supernatant and purified; however, a DNA probe produced from this peptide did not anneal to the genome of *C. piscicola* UAL26. Methods of increasing the production of the peptide and optimizing its purification were examined before a second purification was attempted. A 24 amino acid peptide was then purified and sequenced by Edman degradation. It was identical to a sequence of bovine β-casein which may have been a component of the culture medium. The peptide was synthesized and tested against *Listeria monocytogenes* LI0502 and *Carnobacterium divergens* UAL9 for antibacterial activity. No inhibition was detected.

Acknowledgements

I would like to acknowledge and thank my supervisor Dr. M.E. Stiles for his teaching, guidance, and support during my time in Agriculture Food and Nutritional Sciences. My committee members Dr. M.E. Stiles, Dr. L. McMullen, and Dr. J.C. Vederas were always helpful and committed to my project. Their respective knowledge and experience was an invaluable resource for my research.

I greatly appreciate the help of Dr. L. Z. Yan, Dr. C.M.A.P. Franz, Dr. P. Semchuk, Dr. R. Whittal, and Dr. M. van Belkum for taking the time to teach me the theories and techniques that were central to my project.

The technical assistance of Dr. L. Elmes, Dr. D. Sheehan, N. Rose, M. Johnstone, Mary Mah, Reynold Jaipaul, the staff of Alberta Agriculture Agri-Food Laboratories Branch, Department of Agriculture Food and Nutritional Sciences, Biochemistry, Chemistry and at the Protein Engineering Network Centres of Excellence were very helpful.

I was very happy to have known and worked with Young Nam, Charles, Marco, Lucas, Doug, Yan, Tish, Valerie, Denise, Susan, Jennifer, Nora, Rupi, Julie, Joo eun, Judy, Monique and all the graduate and summer students in the food microbiology lab.

Table of Contents

List of Tables

List of Figures

List of abbreviations

AEC	Anion Exchange Chromatography
API	Alberta Peptide Institute
APT	All Purpose Tween Broth
ATCC	American Type Culture Collection
AU	Arbitrary Units
BHI	Brain Heart Infusion Medium
BBL	Trademark of Becton Dickinson (Cockeysville, MD)
CAA	Casamino acids
CB-1	*Carnobacterium* DNA probe #1
CB-2	*Carnobacterium* DNA probe #2
CEC	Cation Exchange Chromatography
Da	Dalton
EDTA	Ethylamine Diamine Tetraacetic acid
ENSAIA	Ecole Nationale Supérieur d'Agronomie et des Industries Alimentaires
FPLC	Fast Protein Liquid Chromatography
HFBA	Heptafluorobutyric Acid
HPLC	High Performance Liquid Chromatography
HEC	Hydrophobic Exchange Chromatography
HIC	Hydrophobic Interaction Chromatography
IEC	Ion Exchange Chromatography
LAB	Lactic Acid Bacteria
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
MEM	Eagles Minimum Essential Medium
MRS	de Man, Rogosa Sharp medium
PAGE	Poly Acrylamide Gel Electrophoresis
PTC	Phenylthiocarbamyl
PITC	Phenylisothiocyanate
RPMI	Roswell Park Memorial Institute Medium
SDS	Sodium Dodecyl Sulfate
SEC	Size Exclusion Chromatography
SSPE	Sodium chloride, sodium phosphate, EDTA
TE	Tris EDTA
TFA	Trifluoroacetic acid
UAL26	University of Alberta Lactic Acid Bacteria collection, culture no. 26
YE	Yeast Extract

1. General Introduction

Lactic acid bacteria (LAB) are nonsporulating, gram-positive, catalase-negative microaerophilic organisms. Some LAB are capable of fermenting carbohydrates to lactate, acetate, and carbon dioxide (De Vuyst and Vandamme 1994). LAB are generally considered safe for human consumption and they are often used as starter cultures in foods and beverages. In addition to their use as flavor enhancers, LAB are valued for their antagonistic activity toward food spoilage and pathogenic microorganisms.

The potential for LAB to inhibit microbes is attributed to the production of organic acids, hydrogen peroxide, diacetyl, and bacteriocins. In recent years, the use of microorganisms that produce bacteriocins found in fresh meat have been suggested as biopreservatives for meats (Stiles 1996). Currently, nisin which is a lanthionine-containing class I bacteriocin (Nes et al. 1996) is licensed for use as a preservative in over 50 countries. It has a broad spectrum of activity against gram-positive microorganisms and it inhibits the germination of *Bacillus* and *Clostridium* spores. However, this bacteriocin is inactivated by glutathione in fresh meat (Rose et al. 1999b). In an attempt to find a bacteriocin capable of inhibiting and/or killing foodborne pathogens in fresh meat, a number of bacteria from fresh meat have been screened for bacteriocin production (Ahn and Stiles 1990). One strain of LAB, *Carnobacterium piscicola* UAL26, has been shown to have a broad spectrum of activity (Gao unpublished) against both pathogens and spoilage organisms.

Carnobacterium was first described in 1987 as a heterofermentative genus within the LAB that were straight slender rods occurring singly, in pairs or as short chains (Collins et al. 1987). *C. piscicola* was originally known as *Lactobacillus piscicola* or

Lactobacillus carnis (Shaw and Harding 1984). Carnobacteria have been isolated from luncheon meats, chilled chicken meat, fresh fish and chill-stored vacuum-packaged meats (Stiles 1994). *C. piscicola* UAL26 was isolated from vacuum packaged beef by Burns (1987). The bacteriocin produced by *C. piscicola* UAL26 may be active in fresh meat because UAL26 forms part of the normal microflora of the meat. The bacteriocin produced by this organism has a broad spectrum of activity and it is heat stable. Also, the genes for production of this bacteriocin are located on the chromosome (Ahn and Stiles 1992), which may make the bacteriocin more stable.

The objective of this study was to isolate and sequence the antibacterial compound(s) produced by *C. piscicola* UAL26. The genes for production of this bacteriocin were to be located by a process known as reverse genetics. Reverse genetics exploits the universality of the genetic code. Genes are transcribed from a template sequence of deoxyribonucleotide bases into a complementary sequence of ribonucleotides. The sequence of nucleotides is translated into an amino acid sequence. A complementary DNA probe was to be synthesized based on this amino acid sequence. If the probe annealed to its complementary sequence on the genome the gene would have been sequenced.

2. Literature review

2.1 Review of broad-spectrum Class II bacteriocins.

Humans have adventitiously used the preservative and flavor-enhancing capabilities of LAB in foods for thousands of years. The main effect of these organisms has been to lower the pH through the production of lactic acid or lactic and acetic acids which can inhibit the growth of pathogenic and spoilage microorganisms, thereby extending the storage life of perishable foods. The principal antimicrobial factors associated with LAB are organic acids and hydrogen peroxide. Another group of antibacterial compounds (bacteriocins) are produced by some LAB. They were first discovered in 1925 and have been defined as proteinaceous molecules with a bactericidal activity against other strains of the same species (De Vuyst and Vandamme 1994). The antimicrobial peptides produced by LAB have been called lactococcins, carnobacteriocins, leucocins, and pediocins etc. according to the genus of bacteria by which they were produced. In recent years, and with the advent of genetic engineering techniques, bacteriocins have been more intensively studied for use as food preservatives to prevent the growth of spoilage and pathogenic microorganisms.

2.1.1 Classification of bacteriocins.

From the isolation of the first colicin produced by *Escherichia coli* (Gratia 1925) and the first antibacterial substance (bacteriocin) reported to be produced by *Lactococcus lactis* (Rogers 1928), the definition of bacteriocins has undergone several changes. Tagg et al. (1976) suggested six criteria for the definition of bacteriocins of gram-positive

bacteria. Based on these criteria, bacteriocins were defined as: proteinaceous, bactericidal, having specific binding sites on target cells, plasmid-mediated, lethal to the producer cell, and displaying a narrow spectrum of activity. Exceptions to all of these rules for bacteriocins have been reported except for their proteinaceous character. Klaenhammer (1993) described four classes of bacteriocins.

Class I, are small, heat stable, so-called lantibiotics containing post-translationally modified amino acids (e.g., nisin);

Class II, are small heat stable non-lantibiotic antibacterial peptides (e.g., pediocin PA-1/AcH);

Class III, are the large heat labile non-lantibiotic proteins (e.g., helveticin J); and

Class IV, are bacteriocins containing sugar and/or lipid moieties.

Because of the large number and the diversity of the class II bacteriocins isolated and characterized, they have been subdivided.

Nes et al. (1996) subdivided class II bacteriocins into:

Class IIa; bacteriocins such as pediocin PA-1 showing strong antilisterial activity and having homology with other class IIa bacteriocins at the N-terminus of the peptide (i.e., containing the YGNGVXC motif);

Class IIb, two component bacteriocins (such as plantaricin S) whose activity depends on the complementary action of two peptides; and

Class IIc, separates class II bacteriocins by the mechanism of export of the bacteriocin from the bacterial cell. Class IIc are small, heat stable non-modified bacteriocins with *sec*-dependent leader sequences as opposed to double-glycine-type *sec*-independent

leader sequences, which are typical of Class IIa and IIb.

More recently, van Belkum and Stiles (2000) proposed subdividing the class II bacteriocins into six subclasses:

Class IIa contains bacteriocins with four cysteine residues capable of forming two disulfide bridges, e.g., pediocin PA-1;

Class IIb contains bacteriocins with two cysteine residues capable of forming one disulfide bridge in the N-terminal half of the peptide, e.g., leucocin A;

Class IIc contains bacteriocins with one disulfide bridge that spans the N-terminal and C-terminal termini of the peptide and lack the YGNGVXC motif seen in class IIa, e.g., carnobacteriocin A;

Class IId contains bacteriocins with one or no thiol-containing (cysteine) residues and lacking the YGNGVXC motif, such as lactococcin A;

Class IIe; contains two component bacteriocins in which the two peptides act to enhance activity (e.g., lactacin F) or peptides that have little activity alone (e.g., lactococcin G); and

Class IIf contains atypical bacteriocins, such as AS-48, which is cyclical with a head to tail linkage of the N and C termini of the peptide (Martinez-Bueno et al. 1994).

2.1.2 Review of broad spectrum bacteriocins comparable to pediocin PA-1/AcH.

Although most papers presenting novel bacteriocins deal with some aspect of their inhibitory spectrum, Nes and Holo (2000) state that the inhibitory spectrum is becoming less significant because of the following factors: various external factors can interfere

with antimicrobial activity in well diffusion assay; newly discovered bacteriocins are tested for activity against a limited number of target strains and the sensitivity of target strains to a particular bacteriocin can vary up to 100 fold. For use as a biopreservative in the food industry, a bacteriocin that is effective against a broad spectrum of bacteria may be preferable to one that has a narrow inhibitory spectrum. A broad activity spectrum is a bacteriocin that compares with the antibacterial spectrum of pediocin PA-1/AcH. The bacteriocins that are described below are broad-spectrum class II antibacterial peptides produced by LAB.

2.1.2.1 Pediocin PA-1/AcH

Pediocin PA-1 was first reported by Gonzalez and Kunka (1987) and pediocin AcH was purified by Bhunia et al. (1988). Both bacteriocins are produced as 62 amino acid prepeptides and secreted as 44 amino acid peptides. Sequencing of both bacteriocins has shown that they have the same sequence (Motlagh et al. 1992; Lozano et al. 1992). The bacteriocin is stable from pH 2.5 to 10.0 and remains active after heat treatment (121°C for 15 minutes). Pediocin PA-1/AcH is produced by *Pediococcus acidilactici* H which was isolated from fermented sausages (Bhunia et al. 1988). It inhibits species of *Aeromonas, Bacillus, Brochothrix, Clostridium, Lactobacillus, Leuconostoc, Listeria,* and *Staphylococcus.* Pediocin PA-1/AcH has not been licensed for use as a food preservative but its bacterial ferment (which contains the active bacteriocin) has been added to food products (van Belkum and Stiles 2000).

2.1.2.2 Acidocins

Acidocin A is produced by *Lactobacillus acidophilus* TK9201 (Kanatani et al. 1995). The organism was found as a starter culture in fermented milk. It is reported to be active against species of *Lactobacillus, Pediococcus, Streptococcus, Bacillus, Enterococcus, Propionibacterium, Staphylococcus,* and *Listeria.* The bacteriocin is heat stable after treatment at 80°C for 30 minutes. The genetic sequence of the bacteriocin gene is known (Kanatani et al. 1995). The bacteriocin is plasmid-mediated and it is classified as class IIa (Nes et al. 1996) based on a YGNGVXC motif at the N-terminus of the peptide. The immunity region has not yet been defined but a second open reading frame was found downstream of the structural gene.

Acidocins LF221 A and LF221 B were isolated from *L. acidophilus* LF221 by Bogovič-Matijašić et al. (1998). A crude fraction of both peptides showed that bacteriocin activity was stable at 100°C for 30 minutes and at pH 2 to 9. The bacteriocins are claimed to be active against species of *Lactococcus, Pediococcus, Staphylococcus, Enterococcus, Streptococcus, Listeria, Clostridium,* and *Bacillus* species. A partial sequence of both bacteriocins was determined and their molecular size was estimated to be 3500 and 5000 Da., respectively. There was no homology between acidocin LF221 A and other bacteriocins, but there was 26% homology between acidocin LF221 B and brevicin 27 (Benoit et al. 1997). Mixtures of the two bacteriocins showed no complementary activity. The authors suggested that LF221 A and B could be appropriate for probiotic use because they were isolated from microorganisms found within the intestinal tract and they inhibit the opportunistic pathogen *Clostridium difficile.* The

effect of LF221A and LF221B on *C. difficile* has not been tested in vivo.

2.0.2.3 Brochocin-C

Brochocin-C is a well-characterized, broad spectrum, two-component bacteriocin produced by *Brochothrix campestris* ATCC 43754, which is not a lactic acid bacterium; however, the genus *Brochothrix* is a common contaminant and spoilage bacterium of meats (McCormick et al. 1998). It was originally isolated from soil. The bacteriocin has been reported to inhibit species of *Pediococcus, Lactobacillus, Kurthia, Bacillus, Enterococcus, Streptococcus, Staphylococcus, Carnobacterium, Clostridium, and Brochothrix* (Siragusa et al. 1993; McCormick et al. 1998). Brochocin-C has also been reported to inhibit *E. coli* and *Salmonella enterica* serovar Typhimurium after EDTA treatment (Gao et al. 1999). Brochcin-C is a two-peptide bacteriocin containing BrcA and BrcB. The sequence of the genes for brochocin-C is known and both peptides are required for activity (McCormick et al. 1998). Production of BrcA and BrcB is chromosomally mediated and the mature peptides contain 59 and 43 amino acids, respectively. The peptides are stable at 100°C from pH 2 to 9. McCormick et al. (1998) noted that the inhibitory spectrum of brochocin C is similar to nisin yet there is no structural similarity between these bacteriocins.

2.1.2.4 Enterocins

Enterocin A is a well-characterized antilisterial bacteriocin produced by *Enterococcus faecium* DPC1146 (Aymerich et al. 1996). Enterocin A inhibits species of

Lactobacillus, Pediococcus, Enterococcus, Clostridium, Propionibacterium, Listeria, and *Staphylococcus.* Production of this bacteriocin is chromosomally mediated and it is exported by a dedicated secretion mechanism (Aymerich et al. 1996). Characterization of the genes involved in bacteriocin production has shown a structural prepeptide, immunity protein, induction factor, histidine kinase, response regulator, ABC transporter, and an accessory protein (O'Keeffe et al. 1999). The molecular mass of Enterocin A is 4829 Da. and it may have one disulfide bridge.

Enterocin B is a well-characterized bacteriocin produced by *Enterococcus faecium* BFE 900 isolated from black olives (Franz et al. 1996). It acts synergistically with enterocin A (Casaus et al. 1997) and inhibits a wide range of organisms including species of *Staphylococcus, Listeria, Clostridium, Propionibacterium, Enterococcus, Pediococcus* and *Lactobacillus* (Franz et al. 1996). An identical bacteriocin has also been isolated from *E. faecium* T136 and C492 found in fermented sausages (Casaus et al. 1997). Enterocin B is chromosomally mediated. It is an atypical bacteriocin because the immunity region is oriented in reverse direction to the structural gene and there are no regulatory or transport genes clustered near the structural gene (Franz et al. 1999). The molecular weight of Enterocin B is 5479 Da. and it contains one disulfide bond. The bacteriocin is heat stable remaining active after 15 minutes at 121°C and it remains active from pH 2 to 10 (Franz et al. 1996). Enterocin B has 47% homology with Carnobacteriocin A and their prebacteriocins differ at five loci (Nes and Holo 2000).

Enterocin P is a well-characterized, broad-spectrum bacteriocin that is produced by *E. faecium* P13, which was isolated from a Spanish dry fermented sausage (Cintas et al.

1997). The bacteriocin is heat resistant (15 minutes at 121°C) and stable from pH 2 to 11. It inhibits the spoilage bacteria *E. faecalis, Staphylococcus carnosus, Clostridium sporogenes, Clostridium tyrobutyricum,* and *Propionibacterium* spp. and foodborne pathogens including *Listeria monocytogenes, Clostridium perfringens, Clostridium botulinum,* and *Staphylococcus aureus.* The DNA sequence of the gene for the bacteriocin is known. Similar to pediocins (ClassIIa) it has strong antilisterial activity and a YGNGVXC motif at the N-terminus of the peptide but it is secreted from the cell by the general secretory pathway. Enterocin P may serve as an excellent food preservative because it is heat stable, pH stable, it inhibits spoilage and pathogenic organisms, and the producer strain was isolated from a food product.

Enterococcal peptide AS-48 is a well-characterized bacteriocin that is produced by *E. faecalis* subsp. *liquefaciens* S-48 (Galvez et al. 1989). Partially purified bacteriocin was claimed to inhibit species of *Enterobacter, Klebsiella, Proteus, Bacillus, Corynebacterium, Nocardia, Staphylococcus, Micrococcus,* and *Listeria* (Galvez et al. 1986). The mature bacteriocin is cyclical (head to tail) and it contains 75 amino acids. It is strongly cationic with an isoelectric point of 10.5. Based on the gene sequence the prebacteriocin contains 105 amino acids with a 35 amino acid signal peptide (Martinez-Bueno et al. 1994). The mechanism for cyclization is unknown. The bacteriocin forms pores in the membrane of target cells similar to many class II bacteriocins. For a review of the genes involved in AS-48 production and immunity see Martinez-Bueno et al. (1998).

Enterocin L50A (44 amino acids) and L50B (43 amino acids) are two cationic,

hydrophobic bacteriocins produced by *E. faecium* L50 (Cintas et al. 1998). Originally known as pediocin L50 (Cintas et al. 1995), these heat and pH stable bacteriocins have a synergistic effect when combined (Cintas et al. 1998). They do not have an N-terminal leader sequence or signal peptide. These bacteriocins inhibit species of *Lactobacillus, Pediococcus, Leuconostoc, Lactococcus, Enterococcus, Staphylococcus, Clostridium, Listeria, Bacillus* and *Propionibacterium* but not gram-negative organisms (Cintas et al. 1995). As a food preservative, these bacteriocins have several advantages: they are active against a broad spectrum of gram-positive pathogens; they are heat stable and may survive thermal processing; they are active over a wide range of pH; and they are not believed to be post-translationally modified which may make them more amenable to heterologous expression in other microorganisms.

2.1.2.5 Leucocin

Leucocin A-UAL187 is a well-characterized bacteriocin produced by *Leuconostoc gelidum* UAL187, which was isolated from vacuum packaged meat (Hastings et al. 1991). The genes for leucocin A production are plasmid mediated. The bacteriocin inhibits species of *Listeria, Enterococcus, Pediococcus, Carnobacterium, Lactobacillus, Weissella,* and *Leuconostoc.* The DNA sequence of the bacteriocin gene is known and the genes involved in its production have been characterized (van Belkum and Stiles 1995). The bacteriocin contains 37 amino acids with a molecular weight of 3930 Da. and it contains one disulfide bridge. Leucocin A is stable at low pH (2.0) but a reduction in activity occurs above pH 5. Leucocin A is stable at 100°C for 20 minutes but inactivated

in methanol.

2.1.2.6 Thermophilin 13

Thermophilin 13 is a broad spectrum, two-component bacteriocin produced by *S. thermophilus* (Marciset et al. 1997). This two-component bacteriocin inhibits species of *Listeria, Bacillus, Staphylococcus, Micrococcus, Clostridium, Enterococcus, Leuconostoc, Lactobacillus, Propionibacterium,* and *Bifidobacterium.* The first peptide Thm A has a molecular weight of 5776 Da. The second peptide Thm B has a molecular weight of 3910 Da. and has no antibacterial activity. The genetic sequence of both peptides is known. The bacteriocin is antilisterial but lacks a YGNGVXC motif. Thm B enhances the antibacterial activity of Thm A 40 fold but it inhibits the activity of Thm A when Thm B is added in excess. Although brochocin-C is not produced by a LAB, it has a 59% and 49% homology to Thm A and Thm B (McCormick et al. 1998).

2.1.2.7 Bacteriocins that may inhibit gram-negative bacteria.

An interesting group of less well-characterized broad-spectrum bacteriocins may be attractive as antibacterial compounds against gram-negative organisms. Although the production of class II bacteriocins capable of inhibiting gram-negative bacteria such as *E. coli, Pseudomonas* and *Yersinia* is controversial they are beginning to be reported in the literature. The following group of bacteriocins (plantaricin LP84, propionicin PLG-1 and thermophillin 81) may inhibit gram-negative organisms.

Plantaricin LP84 is a less well-characterized bacteriocin produced by *Lactobacillus*

plantarum NCIM 2084 (Suma et al. 1998). The bacteriocin is claimed to inhibit species of *Bacillus, E. coli, Staphylococcus, Pseudomonas* and, except for *L. amylovorus* DSM 20531, this bacteriocin did not inhibit related lactobacilli. Although the bacteriocin was claimed to inhibit gram-negative microorganisms, it was not purified before being tested. The bacteriocin was detected at the end of the exponential growth phase and production was highest at 40°C. The molecular weight of the bacteriocin is between 1,000 and 5,000 Da. as determined by size exclusion chromatography. It was active after heat treatment at 120°C for 20 minutes although heat stability is associated with almost all bacteriocins from *L. plantarum*.

Propionicin PLG-1 is produced by *Propionibacterium* PLG-1 (Lyon and Glatz 1991). It is not a LAB. The bacteriocin is claimed to inhibit species of *Listeria, Pseudomonas, Vibrio, Yersinia* and *Corynebacterium* (Lyon and Glatz 1993). The ability of the bacteriocin to inhibit gram-negative bacteria is questionable because partially purified bacteriocin was used for the activity tests. The effectiveness of propionicin PLG-1 as a food preservative was studied in skim milk (Lyon et al. 1993) and its storage stability was studied at various temperatures (Hsieh and Glatz 1996). When the bacteriocin was added to skim milk and stored at 4°C it retained activity for 14 weeks. Storage at higher temperatures resulted in a gradual loss of activity.

Streptococcus thermophilus 81 produces a bacteriocin (4500 to 5000 Da.) that is claimed to be active against *Bacillus, Listeria, Salmonella, E. coli,* and *Yersinia* (Ivanova et al. 1998). When tested against *E. coli* its action was bacteriostatic. The claim that this bacteriocin is active against gram-negative bacteria is credible because tests were done

with purified bacteriocin. It is a heat labile bacteriocin but it remained active after six months of storage at 40°C. Activity was maintained over a wide pH range (3 to 10) but activity was lost during separation on SDS-PAGE. This bacteriocin is interesting for use as a food preservative because it inhibits gram-negative and gram-positive bacteria. The authors believe that this bacteriocin is ideal for use with yogurt starter cultures because it has very little activity against *Lactobacillus delbrueckii* subsp. *bulgaricus* but it did inhibit strains of *S. thermophilus*. The drawback in applying this bacteriocin to food products is that it is not thermostable and may not survive well in heated foods.
In Table 1, each of the class II broad-spectrum bacteriocins included in this review have been placed into Nes et al. (1996) and van Belkum and Stiles (2000) classification schemes.

2.2 Purification of Class II Bacteriocins.

LAB are fastidious microorganisms and therefore for optimal growth and production of bacteriocins, the LAB are usually grown in broth media which contains several peptides with sizes similar to the bacteriocins of interest (Carolissen-Mackey et al. 1996). Because the growth medium contains a mixture of peptides, proteins, sugars, and lipids, the purification of bacteriocins is complex. There are several complicating factors that can interfere with bacteriocin purification: The amount of bacteriocin produced can be very small, because bacteriocins are active against other bacteria at picomolar to nanomolar concentrations (Nissen-Meyer and Nes 1997); some bacteriocins consist of two component peptides that only produce activity in the presence of each

Table 1. Classification of broad-spectrum class II bacteriocins.

	Nes et al. 1996	van Belkum and Stiles 2000
Acidocin A	A	D
Acidocin LF221 A	C*	D*
Acidocin LF221 B	C*	D*
Brochocin C	B	E
Enterocin A	C	A
Enterocin B	C	C
Enterocin P	A or C	B
AS-48	A	F
Enterocin L50A & L50B	B	F^
Leucocin A	A	B
Leucocin H	B	E
Plantaricin LP84	—	—
Propionicin PLG-1	—	—
Thermophilin 81	—	—
Thermophilin 13	B	E

(—) Not well characterized.
(^) Also a class IIe bacteriocin because the two peptides act in synergy.
(*) The classification is based on a partial N-terminal sequence.

other (Marciset et al. 1997; Blom et al. 1999); and LAB may produce proteases that are released during fermentation (Law and Kolstad 1983). It is not known how effective these proteases are at cleaving a specific bacteriocin.

A peptide is a polymer of amino acids linked by amide bonds and their molecular weight is usually considered to be below 6000 Da. (Wade 1987). The primary amino acid sequence is important in determining the charge of a bacteriocin. Hydrophobic amino acids include tryptophan, alanine, valine, methionine, proline, tyrosine, leucine, isoleucine and phenylalanine. Amino acids with charged side chains include histidine, lysine, arginine, aspartic acid, and glutamic acid. Secondary and tertiary structure within amino acid sequences can also have implications for peptide hydrophobicity and charge. Polypeptides can have similar or different conformations when crystalized or when in solution (Irvine 1997). Although secondary structure may be absent in peptides containing less than 15 amino acids, the potential for secondary and tertiary structure increases as peptide length increases (Hodges and Mant 1991). Folded peptides may display hydrophillic side chains while hydrophobic side chains are hidden within the core of the peptide. In this way, the peptide may have more hydrophillic properties than its amino acid sequence would indicate. Also, depending on the degree of folding, the apparent size of the peptide as measured by various filtration and chromatographic techniques can differ from the predicted size based on the primary sequence. Peptides can also be separated by affinity chromatography using antibody-antigen or metal affinity columns (Josic and Reutter 1991).

Pediocin AcH and pediocin PA-1 have identical sequences but they were isolated by different methods. Pediocin AcH was purified by gel electrophoresis from the supernatant of a 20-hour culture of *P. acidilactici* H (Motlagh et al. 1992). The pH of the culture was adjusted to 6.0 with NaOH. The cells were centrifuged and the supernatant discarded. The cells were washed with deionized water, acidified with HCl and NaCl was added. The cells were again centrifuged and the supernatant was dialyzed against a 1000 MW cutoff dialysis bag. After SDS-PAGE the bacteriocin was sequenced. Pediocin PA-1 was purified by Gonzalez and Kunka (1987). Pediocin PA-1 was grown in MRS broth at pH 6.0 for 18 hours. The cells were removed by centrifugation and membrane filtration. The bacteriocin was precipitated from the supernatant with ammonium sulphate. The precipitate was then dissolved in tris-maleate buffer and dialyzed. The dialyzed bacteriocin was then passed over two ion-exchange columns (DEAE Sephadex A-25 and CM-Sephadex C25).

The organisms for the production of other bacteriocins were grown in different media, at different temperatures, and at different pH to optimize the production and the isolation of the bacteriocin. The medium for the production of enterocin B was supplemented with 1% glucose. *S. thermophilus* was grown at 42°C to optimize production of the bacteriocin. The supernatant for the isolation of pediocin PA-1/AcH, LF221, leucocin H, brochocin C, leucocin A, and enterocin AS-48 was adjusted to neutral pH for production and isolation of the bacteriocin. Ammonium sulphate precipitation was the common method of concentrating bacteriocins in cell supernatant for acidocins A and LF221, enterocins A, L50 and P, leucocin H and pediocin PA-1, whereas

trichloroacetic acid was used to precipitate thermophillin 13.

Acidocin LF221 was isolated by Bogovič-Matijašić et al. (1998) from *L. acidophilus* LF221. The pellet was resuspended in sodium phosphate buffer (pH 5) and passed over a cation exchange column. The active fraction was further purified by reversed phase FPLC on a C-2/C-18 column. The active peptides were eluted from the column with a linear gradient of 20% to 40% 2-propanol. The active fractions were repurified using a narrower gradient of 30% to 40% 2-propanol.

For purification of Enterocin A produced by *E. faecium* (Aymerich et al. 1996) the pellet from ammonium sulphate precipitation was dissolved in sodium phosphate buffer (pH 5) and the solution was passed over a S-Sepharose fast flow cation exchange column. 10% ammonium sulphate was added to the active fraction. The active fraction was passed over a second cation exchange column (Octyl-Sepharose CL-4B column) and eluted with 70% ethanol as the mobile phase. The bacteriocin was diluted with 50 ml of water and purified by reversed phase HPLC. It was eluted with 2-propanol as the organic modifier.

Enterocin L50 was isolated after ammonium sulphate precipitation from the culture supernatant of *E. faecium* followed by cation exchange chromatography, hydrophobic interaction chromatography and FPLC (Cintas et al. 1998). Enterocin P was isolated by a similar method except that after ammonium sulphate precipitation the active fraction was passed through a G-25 size exclusion column to completely remove all traces of ammonium sulphate (Cintas et al. 1995, 1997). The active fraction was applied to a cation exchange column followed by a hydrophobic exchange column and FPLC. It was

eluted with 2-propanol.

Leucocin H was purified from MRS broth (Blom et al. 1999). The culture was grown at pH 6.8 for 20 hours. The cells were removed by centrifugation and the bacteriocin was precipitated with ammonium sulphate. The precipitate was washed with a water:chloroform mixture (100 ml to 5 ml). The precipitate was extracted with 70% 2-propanol. The active fraction was applied to a cation exchange column followed by FPLC on a C-8 column using 2-propanol as the organic modifier.

Thermophilin 13 produced by *S. thermophilus* (Marciset et al. 1997) in M17 media and supplemented with 1% sucrose at 42°C was precipitated with trichloroacetic acid. The pellet was redissolved in 6 M urea, 2 M NaCl, and 200 mM tris-HCl. This fraction was applied to an FPLC column and eluted with acetonitrile as the organic modifier. Other bacteriocins including leucocin A, brochocin C and enterocin B were purified without ammonium sulphate precipitation. Leucocin A produced by *L. gelidum* UAL187 grown in CAA medium containing casamino acids as the nitrogen source (Hastings et al. 1991) was purified by ammonium sulphate precipitation, hydrophobic interaction chromatography, cation exchange chromatography and reversed phase HPLC with a C-18 column.

Brochocin C is produced by *B. campestris* ATCC 43754 (McCormick et al. 1998). The producer strain was grown in the more defined CAA medium (Hastings et al. 1991) at a constant pH of 6.7. The cells were removed by centrifugation and the bacteriocin was extracted twice with n-butanol. The butanol was removed by vacuum evaporation and the bacteriocin was precipitated with acetone and held at 5°C for 24 hours. The

precipitate was redissolved in 0.1% trifluoroacetic acid and passed over a G-50 size exclusion column. Active fractions were lyophilized and purity was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Attempts at purification using reversed phase HPLC have not yet been successful in separating Brc A from Brc B (Gao unpublished).

Enterocin B is produced by *E. faecium* BFE900 (Franz et al. 1999). The supernatant was passed over an XAD-8 hydrophobic exchange column followed by a cation exchange column. The active fraction was desalted in a Sep Pak column, freeze-dried and purified by C-18 reversed phase HPLC.

Enterocin AS-48 is produced by *Enterococcus faecalis* subsp. *liquefaciens* S-48 (Galvez et al. 1989). The bacteriocin was isolated from the culture supernatant of BHI broth and adjusted to pH 6.2. Cation exchange resin was then added to the supernatant and stirred. The supernatant was discarded and the resin was washed with sodium phosphate buffer. The resin was transferred to an empty column and the bacteriocin was eluted with a linear gradient of NaCl (0 to 2 M). After a second cation exchange step, the active fraction was purified by C-4 reversed phase chromatography and eluted with a 0 to 100% linear gradient of isopropanol:acetonitrile (2:1 vol/vol) mixture.

The common aspect to the isolation of the broad-spectrum bacteriocins was that final purification was achieved by HPLC or FPLC. FPLC allows for the separation of peptides using low pressure but offers one advantage over HPLC in that the sample does not have to be desalted before being added to the column.

2.3 Potential of broad spectrum bacteriocins for food preservation.

A major goal of searching for and characterizing bacteriocins is to enhance the safety of the food supply. There are many criteria both regulatory and scientific that must be overcome to produce a bacteriocin-based system capable of protecting the consumer against foodborne pathogens. The introduction of new preservatives into the market requires the approval of regulatory agencies. Organisms that have been associated with human food and consumed by humans for several years without showing pathogenicity have been given GRAS (Generally Recognized As Safe) status and may more easily get approval from regulatory agencies. Many antibacterial compounds such as lactic and acetic acids have this designation. Although the LAB that produce bacteriocins have been consumed for thousands of years, other than nisin, bacteriocins produced by LAB represent an untapped resource for preservatives in the food supply.

Of the broad-spectrum bacteriocins reviewed, each has advantages and disadvantages associated with it. When searching for a bacteriocin to be used in a specific food product, organisms found in that environment may be more effective at killing pathogens associated with that habitat. Thermophilin 81 and acidocin B affect pathogens found in their respective niches (yogurt and cheese) without necessarily affecting the producer strains. Therefore, these bacteriocins may be easier to work with when developing a preservative for use in certain food products. Genes found on the chromosome such as the bacteriocin produced by *Carnobacterium piscicola* UAL26 (not yet isolated) may be more genetically stable than those found on plasmids. This means that the producer organism may not transfer the bacteriocin gene to another organism as

easily or lose bacteriocin production due to the loss of the plasmid. Another problem associated with bacteriocins is the development of resistance amongst the target organisms. The common foodborne pathogen *L. monocytogenes* develops resistance to AS-48, mesentericin 52, curvacin 13 and plantaricin C19 and when resistance develops to mesentericin 52, curvacin 13 and plantaricin C19 it is a stable characteristic (Mendoza et al. 1999; Rekhif et al. 1994). Combining bacteriocins can increase activity (Hanlin et al. 1993) and may limit the development of resistance if their mechanisms of action are different (Gao unpublished). The discovery of class II bacteriocins that inhibit gram-negative bacteria is surprising because class II bacteriocins are typically thought only to inhibit gram-negative bacteria once the outer membrane has been disrupted. For example, brochocin C has been shown to inhibit *Salmonella enterica* serovar Typhimurium once the outer membrane has been disrupted by a chelating agent such as EDTA (Gao et al. 1999). Thermophilin 81 (Ivanova et al. 1998), enterocin CCM4231 (Laukova et al. 1993), and plantaricin LP84 (Suma et al. 1998) were claimed to inhibit gram negative bacteria; however, these bacteriocins are not well characterized and their bacteriocins have not been synthesized, sequenced, or cloned into a non-bacteriocin producer and tested against gram-negative bacteria.

2.4 Review of casein as an antibacterial agent.

Milk proteins are comprised of two groups. The first group consists of whey proteins, albumin and globulins. The second group of milk proteins (casein) comprises 80% of the protein found in milk (Kunji et al. 1996). Casein is a complex of proteins in

milk (αs_1, αs_2, κ , and β) that differ between mammalian species (Miller et al. 1990).

The common antibacterial components of milk protein include immunoglobulins (antibodies that potentiate the destruction of specific bacteria), lactoferrin that selectively binds iron therefore inhibiting the growth of bacteria, lactoperoxidase (an enzyme that produces hydrogen peroxide), and lysozyme (an enzyme that degrades the cell wall of gram-positive bacteria) (Reiter and Harnulv 1984; Lahov and Regelson 1996). Peptides derived from casein have been claimed to have opiate, immunomodulating, anti-aggregating, anti-thrombotic, anti-hypertensive and antibacterial properties (Fiat et al. 1993).

Specific peptides from casein (Katchalsky et al. 1973; Zucht et al. 1995) have also been reported to have antimicrobial activity. LAB that are used as starter cultures are capable of degrading casein. Many of these bacteria are multiple amino acid auxotrophs (Chopin 1993) and, because the amount of free amino acids and peptides in milk is low (Kunji et al. 1996) the proteolytic degradation of casein is essential for the growth of these bacteria in milk. LAB produce specific enzymes that are capable of cleaving the four types of casein. For a complete review on the proteolytic systems of LAB associated with casein see Kunji et al. (1996).

Lahov (1972) digested casein with rennet and isolated a compound from casein that exerted a protective effect in animals against infection by *Staphylococcus aureus, Streptococcus pyogenes* and *Listeria monocytogenes.* The antibacterial compound was named isracidin and consists of the first 23 amino acids of αs_1 casein (Lahov and Regelson 1996). Katchalsky et al. (1973) also isolated a group of peptides from a digest

of casein (rennet at pH 6) which were collectively called casocidin. These peptides inhibited staphylococci, *Sarcina*, *Bacillus subtilis*, *Diplococcus pneumoniae* and *S. pyogenes* (Lahov and Regelson 1996; Katchalsky et al. 1973).

Zucht et al. (1995) isolated casocidin-I, a 39 amino acid peptide (position 165 to 203 of αs_2 casein) that inhibited the growth of *Escherichia coli* and *Staphylococcus carnosus*. The peptide was synthesized to prove its antibacterial capabilities.

3. Materials and Methods

3.1 Bacterial strains.

C. piscicola UAL26 was isolated from vacuum packaged chilled meat by Burns (1987). This strain inhibits the growth of *Listeria*, *Enterococcus*, *Lactococcus* and *Brochothrix* species (Gao unpublished). Stock cultures of the indicator organisms used in the study were stored at -70°C in APT (All Purpose Tween; Difco Laboratories Inc., Detroit, USA) with 15% glycerol added as a cryoprotectant. Indicator strains *L. monocytogenes* LI0502 (Facultad de Veterinaria, Madrid, Spain), *C. piscicola* UAL9 (University of Alberta lactic acid bacteria collection, culture no. 9, Edmonton, Canada), *B. campestris* ATCC 43754 (American Type Culture Collection, Rockville, Maryland.), *Lactococcus lactis* ATCC 11454, and *Enterococcus durans* ENSAIA 630 (Ecole Nationale Supérieur d'Agromomie et des Industries Alimentaires, Nantes, France) were obtained from the University of Alberta Lab culture collection.

3.2 Method for determining activity.

Activity was tested by spot-on-lawn technique (Tagg et al. 1976). Indicator cultures were subcultured into APT broth at 25°C for 24 hours before use. A 1% inoculum of a fully-grown indicator culture was added to APT soft agar (0.75% agar) that had been melted and stored at 45°C. The inoculated soft agar was then poured onto a prepoured APT plate (containing 1.5% agar) and allowed 10 minutes to solidify. Solutions containing bacteriocin were diluted by doubling dilution with distilled water, spotted onto the indicator plate, and allowed to dry for 30 minutes in a laminar flow hood. The

indicator plates were incubated at 37°C and examined after 24 hours. Clear zones showing no growth of the indicator (*L. monocytogenes* LI0502) were considered to contain antibacterial compound(s).

3.3 Purification scheme for the bacteriocin and genomic probing.

A 1% inoculum of *C. piscicola* UAL26 was grown in 1.4 L of APT broth incubated at 30°C. The cells were removed by centrifugation at 8000 x *g* for 10 min. The culture supernatant was heated to 85°C for 10 minutes and cooled to 4°C in 1 hour. The supernatant (1.4 L) was added to an Amberlite XAD-8 column (8 x 45 cm; BDH Chemicals Ltd., Chicoutimi, Quebec). The column was successively eluted with 2 L of 0, 20, 40, and 80% ethanol in 0.1% trifluoroacetic acid (TFA; Sigma Chemical Co., St. Louis, M.O.). The active fraction (in 40% ethanol) was concentrated to 400 ml by vacuum evaporation at 35°C. The pH was adjusted to 5.0 with 1.0 M ammonium hydroxide. An SP Sepharose 50 Fast Flow (Pharmacia, Uppsala, Sweden) cation exchange column was pre-equilibrated to pH 5.0 with 20 mM sodium acetate. The concentrated active fraction was then added to the column and eluted with 100 ml each of 20 mM sodium acetate (pH 5.0) solutions containing 0, 80, 500, and 1000 mM NaCl. The active fraction was desalted by hydrophobic exchange in a Sep pak (Waters, Milford, MA) column. Similar to hydrophobic exchange on the XAD-8 column the active fraction was eluted with 20 ml volumes of 0, 20, 40, and 80% ethanol (in 0.1%TFA). The volume of the active fraction was reduced by speedvac (SAVANT SC 100; Farmingdale, N.Y.) to 2 ml. One ml of this fraction was applied to a C-18 HPLC column (4.6 mm x 25 cm;

Zorbax SB-C8 Torrance, CA) using a Varian 5000 pump (Varian Inc., Walnut Creek, CA). Mobile phases A and B were acetonitrile/0.05% TFA and water/0.05% TFA, respectively. The gradient was set for 100% to 90% A (0 to 10% B) in 10 min and from 90% to 20% A (10 to 80% B) in 180 min. Flow rate was 1 ml/min and 1 ml samples were collected every minute. The purity of these samples was determined by MALDI-TOF MS (Bruker; Billerica, MA) using sinapinnic acid (Sigma) as the matrix.

3.4 Detection of sample purity by MALDI-TOF MS.

Samples (2 µl) were mixed with equal volumes of saturated sinapinnic acid (Sigma) and allowed to dry for 10 minutes. Fresh matrix was mixed for each series of tests. Matrix consisted of 200 µl of 0.1%TFA, 400 µl of acetonitrile, and 50 mg of sinapinnic acid (Sigma). The conditions for MALDI-TOF MS analysis were similar to Rose et al. (1999a) except that the probes were not washed with water after the samples were dried. The MALDI-TOF MS instument was calibrated with β-insulin (Sigma). The MALDI-TOF MS analysis was based on 60 readings (shots) of each sample.

3.5 Isolation of genomic DNA from UAL26.

C. piscicola UAL26 genomic DNA was isolated by the method of Quadri et al. (1994). A 1% inoculum of UAL26 was grown in APT broth at 25°C for 16 hours. The cells were centrifuged at 8000 x *g* for 10 min, washed with 0.5% NaCl and resuspended in 3 ml of 25% sucrose, 50 mM Tris-HCl (Sigma) and 5 mM EDTA (Sigma) at pH 8.0. Sixty mg of lysozyme (Sigma) was added and the solution was incubated at 37°C. After

1 hour, 3 ml of 1% SDS (Sigma), 100 mM EDTA (Sigma) was added and the solution was incubated at 65°C for 15 minutes. Proteinase K (0.6 ml of 1 mg of proteinase K/ml; Sigma) was added; the solution was incubated at 56°C overnight, and 0.6 ml of 1 M NaCl was added. The DNA was extracted twice with two volumes of phenol and one volume of chloroform. The DNA was precipitated with 95% ethanol and washed with 70% ethanol. The DNA was vacuum dried and 200 µl of TE buffer (pH 8.0) was added. One µl of RNase (10 mg/ml; Sigma) was added.

3.6 Probing of genomic DNA of UAL26.

Southern blot and probing of genomic DNA was done on Hybond N (Amersham, Oakville, ON) nylon membranes using the well-established methods of Sambrook et al. (1989). Oligonucleotides CB-1 5'AA(AG)CA(CT)CC(ACTG)AT(ACT)AA(CT)AT(ACT)CC3' and CB-2 5'AAACATCC(AT)AT(AT)AATAT(AT)CC3' were end-labeled with γ^{32} P (Amersham, St. Catherines, ON). Hybridizations were done in 6 x SSPE with 5 x Denhardt's and 0.5% SDS at 60°C. The membranes were washed with 0.6 x SSPE, 0.5% SDS at 50°C. The stringency for hybridization was reduced by reducing the temperature of hybridization (Sambrook et al. 1989).

3.7 Optimization of bacteriocin production by UAL26.

C. piscicola UAL26 was subcultured from 15% glycerol:APT stock solution between two and six times in APT broth at 25°C for use in experiments. For growth rate

determinations, a 0.1% inoculum of a 24-hour culture was added to 200 ml of APT broth and shaken at 200 rpm at 25°C. Duplicate samples were collected at 6, 12, 14, 16, 18, 21, 23 and 24 hours. A second flask of APT broth was inoculated and held static to serve as a control. Growth rates of the test culture were determined by optical density at 600 nm (Spectronic 21, Spectronic Instruments Inc., Cambridge, England). For productivity at varying pH levels, UAL26 was grown in APT broth adjusted to pH 6.0, 7.0 and 8.0 with tris-HCl (1 M). pH was maintained with 1 M NaOH using a Chem-cadet controller (Cole Parmer, Chicago, Ill.).

Duplicate 1 ml samples of all conditions of all aeration, pH, and temperature broths were taken at 6, 12, 14, 16, 18, 21, 23 and 24 hours from two replicates of each test. The volume was reduced by Speedvac (Savant, Farmingdale, NY) to 250 µl (a two-fold reduction) and 10 µl of this solution was spotted onto indicator plates containing *L. monocytogenes* LI0502. Solutions of uninoculated broth and *C. divergens* UAL9 inoculated into APT broth were treated in the same manner and used as controls.

To test the effects of nitrogen source on *C. piscicola* UAL26 bacteriocin production, UAL26 was grown on duplicate 1.5% agar plates and tested by deffered inhibition (Tagg et al. 1976) against *L. monocytogenes* LI0502. The plates were made using ingredients from Difco (Tryptone, Soytone, Casitone, Casamino acids, Peptone, Proteose peptone, and Yeast Extract) and Yeast Extract from BBL (Microbiology Products, Cockseyville, MD). A second set of plates containing Yeast Extract (Difco) with one of Tryptone, Soytone, Casitone, Casamino acids, Peptone, or Proteose Peptone were also poured and tested. The plates were incubated at 30°C overnight and examined for zones of

inhibition. The effect of increasing yeast extract concentration on bacteriocin production was determined with different concentrations (3.75, 7.5,15, 30 and 60 g/l) of Yeast Extract in APT agar (Difco). Another set of plates containing tissue culture medium; MEM (Eagles Minimum Essential Medium), RPMI (Roswell Park Memorial Medium), and Williams E Medium were also tested in the same manner for growth and production of bacteriocin by UAL26. The tissue culture media were aquired from Gibco Life Technologies, Gaithersburg, Maryland.

3.8 Optimization of the purification of the bacteriocin produced by UAL26.

3.8.1 Hydrophobic exchange of bacteriocin on an XAD-8 column.

C. piscicola UAL26 was grown overnight at 30°C in 2 L of APT. The cells were centrifuged for 10 min at 8000 x *g*. The supernatant was applied to an Amberlite XAD-8 column (10 x 20 cm; BDH Chemicals Ltd., Poole, England) and washed with 3 L of 0.1%TFA. Successive washes of 20, 25, 30, 35, 40, 50, and 60% ethanol in 0.1%TFA were assayed for activity by spot-on-lawn technique. The tests were performed in duplicate.

3.8.2 Cation exchange of bacteriocin on a Sepharose column.

A fast flow SP sepharose column (Pharmacia, Uppsala, Sweden) was pre-equilibrated with 20 mM sodium acetate pH 5.0. UAL26 was grown in 1 L of APT broth at 25°C. The cells were removed by centrifugation at 8000 x *g* for 10 min. The supernatant was passed over an XAD-8 column and eluted with 2 L each of 20, 40 and

80% ethanol. The active fraction was concentrated to 250 ml by vacuum evaporation at 35°C and 10 ml of the concentrated supernatant was adjusted to pH 5.0 with sodium acetate buffer. The sample was then added to the sepharose column. The column was washed with 0, 50, 100, 150, 200, 300, 400, 500 and 1000 mM NaCl (60 ml of each) at a flow rate of 3.5 ml/min. Samples of 10 µl were spotted onto lawns of *L. monocytogenes* LI0502. Salt solutions used to wash the column were also spotted onto the indicator lawn as controls.

3.8.3. Optimization of High Performance Liquid Chromatography.

Samples containing bacteriocin were injected into an HPLC column and separated by altering the composition of the mobile phase for gradient elution. The solvents used were HPLC grade water (Fisher; Edmonton, AB), acetonitrile (Fisher), methanol (Fisher), and 2-propanol (Fisher). Ethanol (Fisher) was not denatured. Trifluoroacetic acid (Sigma) was added to both mobile phases A and B unless otherwise stated. All solutions were degassed by sonication for 20 minutes or by vacuum filtration. The columns used in the separation were C-4, C-8, and C-18 VYDAC (4.5 mm x 25 cm; Hesperia, CA) reversed phase analytical columns. The HPLC pump was a Varian 5000 (Walnut Creek, CA). The samples were detected by a Waters 486 detector (Milford, MA) at 212 nm and at 230 nm for solvents containing ethanol. The column heater was a Biorad (Hercules, CA) HPLC column heater. The tubing was analytical grade. Mobile phase A was the inorganic phase and mobile phase B was the organic phase.

Column washing involved flushing the column with mobile phase A (flow rate 1

ml/min) for 5 min then increasing the gradient to 80% of B in 5 minutes. Mobile phase B was sustained at 80% for 10 min followed by 100% of A for 10 minutes. One ml of active component containing 12,800 AU/ml from UAL26 was added for each test except for runs involving increases in temperature in which 1 ml of active component containing 3200 AU/ml was added. Tests were also done by changing the gradient from 10 to 80% of B at 0.45%/min, 0.90%/min, and 1.80%/min. When testing different counter ions, phosphoric acid or sodium acetate, these solutes were added to the inorganic phase only. The flow rate for all of the samples was 1.0 ml/min but the flow rate for 2-propanol was adjusted to 0.5 ml/min. All chromatography tests involving flow rate, gradient, temperature, and changing organic modifiers were done a minimum of ten times.

The concentration of the bacteriocin in fractions taken from the HPLC column were determined by spot-on-lawn technique. The amount of activity was measured in arbitrary units (AU), which was the reciprocal of the last dilution that produced a clear zone of inhibition against the indicator *L. monocytogenes* LI0502 multiplied by 100 (Franz 1999).

3.9 Effect of enzymes, pH, and heat on bacteriocin activity.

The stability of the bacteriocin produced by *C. piscicola* UAL26 was tested using fractions of cell supernatant from fully grown cultures of BHI broth (Difco)(sample **A**), partially purified bacteriocin (sample **B**), and dialyzed, partially purified bacteriocin (sample **C**). Sample **A** was prepared by freeze-drying 250 ml of supernatant that eluted with 40% ethanol from the XAD-8 column. Partially purified bacteriocin (sample **B**) was obtained by applying 1 ml of sample **A** to a C-18 reversed phase HPLC column with 2-

propanol as the organic solvent. The elution was run at room temperature (0.5 ml/min) with a 10 to 80% gradient mobile phase A (water, 0.1%TFA) to B (2-propanol, 0.1%TFA) over 60 minutes. The dialyzed, partially purified bacteriocin (sample **C**), was obtained by combining 3 ml of sample **B** and dialyzing it against 400 ml HPLC grade water (Fisher) containing 0.1%TFA for 3 hours with one change of buffer.

Solutions containing 1 mg/ml of each enzyme (trypsin, chymotrypsin, or Protease K; Sigma; St. Louis, MO) were prepared by dissolving each enzyme in 50 µl of MilliQ water. A 10 µl fraction of samples **A**, **B** or **C** was mixed with 10 µl of each enzyme solution. After 10 minutes, 5 µl was tested for activity by spot-on-lawn technique. For a control, each sample was mixed with an equal volume of HPLC grade water (Fisher) and tested by spot-on-lawn technique as a control.

To test the sensitivity of the bacteriocin to pH and heat, 100 µl of sample **A** was mixed with 100 µl of tris-HCl buffer at pH 2, 4, 7, 8, or 9 and stored at room temperature for three hours. Twenty µl portions of samples at pH 2, 4, 7 and 8 were held at -20, 4, and 25°C for 30 days and assayed for activity. Samples (20 µl) from pH 2, 4, 7 and 8 were heated to 85°C for 30 minutes and assayed by spot-on-lawn technique for activity against *L. monocytogenes* LI0502 after 30 days storage at 4°C.

3.10 Optimized purification scheme.

A 1% inoculum of *C. piscicola* UAL26 was grown in 10 L of BHI broth (Difco) at room temperature (pH 8.0). After 16 hours, the cells were centrifuged at 8000 x *g* for 10 min. The supernatant was added to an XAD-8 column and washed with 25% ethanol

0.1% TFA (3 L). The bacteriocin was eluted with 40% ethanol in 0.1%TFA (3 L). The ethanol was removed by drying under vacuum at 35°C and the sample was freeze-dried to a volume of 20 ml. A 5 ml sample was added to a Sephadex G-50 (2.5 x 120 cm Pharmacia, Piscataway, NJ) size exclusion column. The bacteriocin was eluted at a flow rate of 0.4 ml/min and the mobile phase was 0.1%TFA. The size exclusion column was run four times and the active fractions were collected and combined. A volume of 400 ml of active solution was recovered and it was added to a cation exchange column (SP sepharose) pre-equilibrated with 20 mM sodium acetate (pH 5.0). The column was washed with 100 ml of 150 mM NaCl in 20 mM sodium acetate (pH 5.0). The active component was eluted with 200 mM NaCl in 20 mM sodium acetate (pH 5.0). A volume of 40 ml of active component was recovered. The active component was desalted with a Sep pak (Waters, Milford, MA). It was eluted with 40% ethanol. The 5 ml sample was freeze-dried to a volume of 1 ml and added to a C-18 reversed phase HPLC column. The column was prepared as described for HPLC optimization (Section 3.8.3.). The inorganic phase was 0.05% TFA (solution A) and the organic phase contained 0.05% TFA:2-propanol (solution B). The column was run from 100% to 90% A (0 to 10% B) in 10 min and 90% to 20% A (10% to 80% B) in 80 min. Flow rate was 0.5 ml/min and the column was heated to 65°C. The active fraction was recovered at 43 min (10 µl was saved for purity testing). The active component was then re-injected into the column under the same conditions except that 100% anhydrous ethanol was used as the organic solvent and the flow rate was increased to 1 ml/min. The active compound was eluted at 64 min (1 ml) and freeze-dried. It was dissolved in 100 µl of sterilized HPLC grade

water (Fisher) and sent to the Alberta Peptide Institute (API; Edmonton, AB) for N-terminal amino acid sequencing and 20 µl was saved for purity testing.

Five µl of the active fraction that was isolated from BHI broth was mixed with 5ul of Proteinase K (1 mg/ml) and tested by spot-on-lawn technique for activity against *L. monocytogenes* LI0502. Inactive fractions adjacent to the active fractions were also tested by spot-on-lawn technique.

3.11 Testing the activity of the synthesized peptides.

Three peptides were synthesized by the American Peptide Company (Sunnyvale, CA) for CanBiocin Inc. (Edmonton, AB): Peptide 1 RPKHPINIPPLTQTPVVVPPFLQPEV, peptide 2 SLPQNIPPLTQTPVVVPPFLQPEV, and peptide 3 NIPPLTQTPVVVPPFLQPEV. The three peptides were diluted to 1 mg/ml in 1 ml solutions of water, water:40% ethanol, 0.1% TFA, or 0.1%TFA:40% ethanol. Both water and ethanol were HPLC grade. A 10 µl sample of each solution was tested for activity by spot-on-lawn technique and each test was performed in duplicate. The indicators used were *L. monocytogenes* LI0502 and *C. divergens* UAL9. The controls were solvent solutions without added peptide. A solution of 50 mg/ml (in HPLC grade water) of peptide 2 was also prepared and tested by spot-on-lawn assay.

4. Results

4.1 Isolation of the first peptide.

The first peptide was isolated from APT broth incubated at 30°C (Table 2). The active fraction was eluted from the XAD-8 column in the mobile phase containing 40% ethanol. After cation exchange, there was an 80% loss in activity of the bacteriocin. After the removal of NaCl by Sep pak there was a 97.5% loss in activity. The desalted sample was passed over a C-18 HPLC column. Eluant was collected every minute. Samples 99 through 135 minutes corresponded with peaks on the U.V. detector and were tested for antibacterial activity by spot-on-lawn technique. Samples 110 to 117 gave the largest zones of inhibition when tested on indicator plates overlayed with *L. monocytogenes* LI0502. The activity remaining after HPLC was not titred in order to maximize the amount of peptide available for amino acid sequencing by Edman degradation.

The results of the HPLC of the first peptide (Figure 1) isolated from APT broth indicate a single peak of higher intensity than all others at approximately 115 min. Activity was detected in samples 103 to 125 min; therefore, activity corresponds with the most intense peak of peptide in the sample.

The MALDI-TOF MS data for the first peptide shows that the sample contained two compounds (Figure 2), with peaks detected at 2892 and 5690 Da. Matrix adduct peaks were detected at 3100 and 5900 Da (Dr. R. Whittal, personnal communication). MALDI-TOF MS analysis of (active) samples adjacent to the sample at 112 minutes also had a peak at 2900 Da. with lower intensity; however, these samples were contaminated

Table 2. Volume, activity and percent recovery of antimicrobial compound of the first antibacterial peptide isolated from *C. piscicola* UAL26 grown in APT broth at 30°C.

Fraction	Volume	Activity AU/ml	%Recovery
Supernatant	1.4 L	—	—
Hydrophobic exchange XAD-8	2 L	—	—
After Drying	0.4 L	400	100%
Cation exchange SP Sepharose	0.04 L	800	20%
Desalting Sep pak + speedvac	0.002 L	400	0.5%
HPLC Organic modifier Acetonitrile	0.001 L	—	—

(—) Not determined.

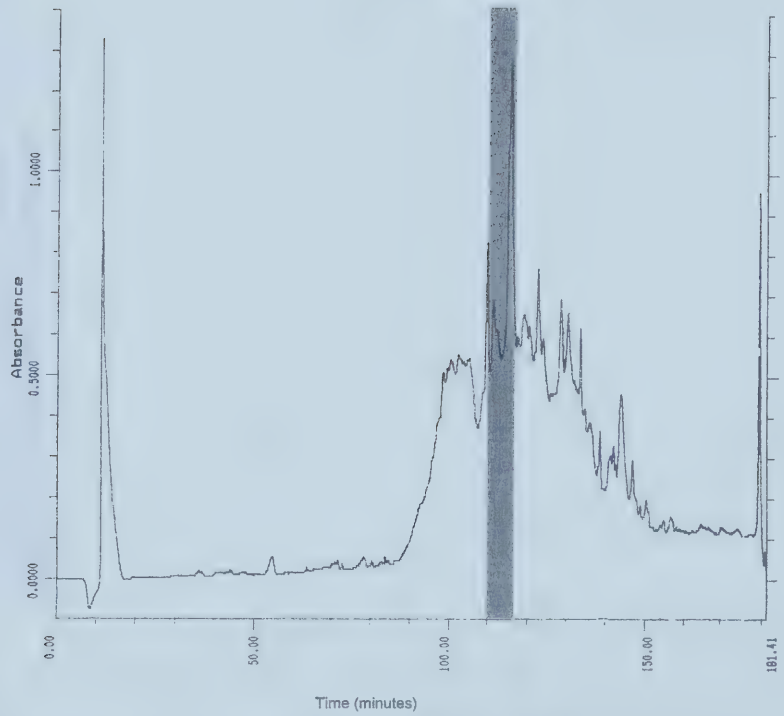

Figure 1.

HPLC elution pattern of the first compound isolated from C. piscicola UAL26. The organic modifier was acetonitrile with a flow rate of 1 ml/min at room temperature. Fractions 99 to 137 were collected every minute and assayed for activity against L. monocytogenes LI0502. Fractions 110 to 117 (▬) contained active antibacterial compound against L. monocytogenes LI0502.

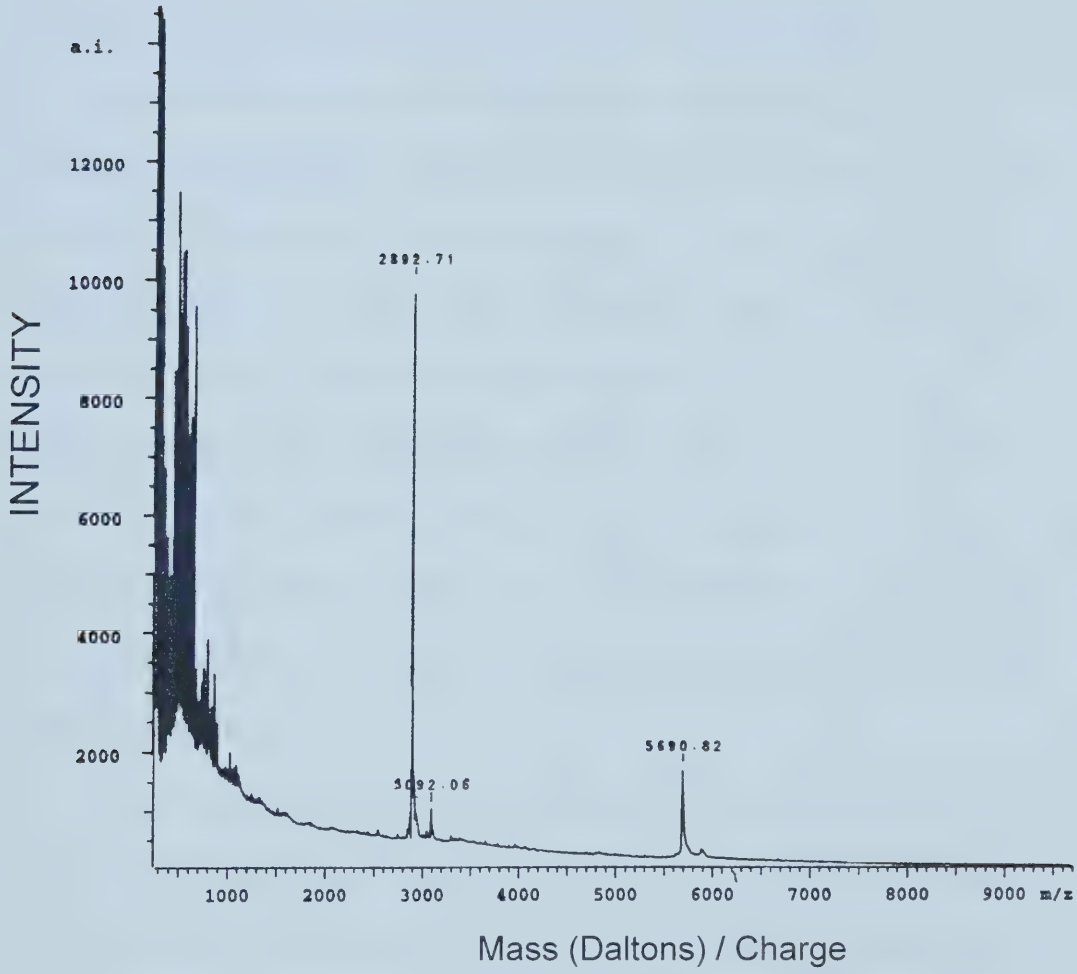

Figure 2.

MALDI-TOF MS spectrum of the first peptide isolated from *C. piscicola* UAL26 grown in APT broth.

with other peptides (based on MALDI-TOF MS analysis) and discarded.

4.2 Probing the UAL26 genome based on the sequence of the first peptide.

The sequence that was obtained did not show any contaminating peaks during Edman degradation; therefore, the second peak was believed to be a dimer or an artifact of MALDI-TOF MS analysis. Given this knowledge, the peptide was presumed to originate from UAL26 and a DNA probe was synthesized to detect the bacteriocin gene using reverse genetics. UAL26 does not contain plasmids (Ahn and Stiles 1992), therefore the bacteriocin gene should be located on the genome. Neither of the DNA probes, CB-1 nor CB-2, annealed to the template DNA on the Southern Blot. Less stringent annealing temperatures (60°C, 55°C, 50°C, and 45°C) did not show binding to the template DNA on the Southern Blot. At 40°C, nonspecific binding, of both probes to the *Cla*I lambda DNA ladder occurred.

The growth medium (APT) for UAL26 contained casein hydrolysate. It was assumed that the peptide that was isolated was a portion of casein found in the medium because it is unlikely that UAL26 produces a peptide with a sequence of amino acids identical to bovine casein. Before a second isolation was attempted, methods of improving the production and purification of the bacteriocin were examined.

4.3 Optimization of bacteriocin production from UAL26.

4.3.1 Effect of enzymes, pH, temperature and solvents on bacteriocin stability.

The partially purified active compound from samples **A**, **B**, and **C** (see section 3.9)

Table 3. Effects of enzymes, pH, temperature, and solvents on UAL26 bacteriocin activity.

Treatment		Activity
Untreated	Samples A, B, and C	+
Enzymes:		
Trypsin	Sample A	--
	Sample B	--
	Sample C	--
Chymotrypsin	Sample A	--
	Sample B	--
	Sample C	--
Proteinase K	Sample A	--
	Sample B	--
	Sample C	--
pH: Sample A		Activity (AU/ml)
Sample A		400
2		400
4		400
7		400
8		400
9		200
Temperature: Sample A		
-20°C		400
4°C		400
25°C		400
85°C (30 min)		400
Solvent: Sample A		
Methanol		0
Ethanol		400
2-propanol		400
Acetonitrile		400
Water		400

Sample A: Freeze-dried cell supernatant from UAL26 (Section 3.9).
Sample B: HPLC partially purified bacteriocin in 2-propanol (Section 3.9).
Sample C: HPLC partially purified bacteriocin in water (Section 3.9).
AU is defined as the reciprocal of the highest dilution showing a clear zone of inhibition and multiplied by 100 to obtain the AU/ml.

were tested for activity after exposure to treatments similar to the conditions used during purification of the bacteriocin. The data in Table 3 indicate the stability of the bacteriocin to treatment with various enzymes, heat, pH, and to the effects of solvents. Activity against the indicator *L. monocytogenes* LI0502 was abolished when the bacteriocin was exposed to trypsin, chymotrypsin, and proteinase K indicating the proteinaceous nature of the active compound.

The bacteriocin was stable in samples maintained at –20, 4, and 25°C for thirty days and when adjusted to pH 2, 4, 7, and 8. At pH 9 there was a 50% loss of activity (Table 3). The bacteriocin was also stable when heated to 85°C for 30 min at pH 5.0. There was no loss of activity when sample **A** was stored for 30 days in ethanol, 2-propanol, acetonitrile, or water; however, activity was lost when the bacteriocin was stored in methanol.

4.3.2 Effect of nitrogen source on bacteriocin production.

C. piscicola UAL26 was grown in APT and BHI broths. These are complex media containing nitrogen from a number of sources. In an attempt to find a more defined medium for growth and production of bacteriocin by UAL26, the organism was grown in tissue culture media MEM, RPMI, and Williams E. Because no activity was detected in these tissue culture media (results not shown), a component of APT broth, yeast extract (YE), a complex yet more defined medium than APT, was tested to determine if UAL26 would produce bacteriocin when grown in this medium. Once it was determined that UAL26 produced activity against *L. monocytogenes* LI0502 when grown in Yeast Extract

(YE), increasing bacteriocin production by supplementing YE was investigated. UAL26 grew within 24 hours on each of the media tested (see Table 4). The largest zones (>12 mm) of inhibition were observed on plates containing YE from Difco and YE (Difco) with Proteose Peptone. A larger zone of inhibition was seen when UAL26 was grown on YE obtained from Difco than when it was grown on YE obtained from BBL. The method of production of these media is proprietary. APT broth (Difco) was used as a control.

Because none of the supplemented media (Tryptone, Soytone, Casitone, Casamino acids, Peptone, or Proteose Peptone) produced activity (see Table 4), increasing the concentration of YE was tested (see Table 5). Once YE concentration reached levels found in APT agar there was no increase in bacteriocin production indicating that the level of YE found in APT was optimal for bacteriocin production. At YE levels above 30 g/l no activity was detected. APT broth (Difco) was used as a control.

4.3.3 Effect of aeration, temperature and pH on bacteriocin production by C. piscicola UAL26.

Because aeration and temperature may affect bacteriocin production (Coventry et al. 1996; Callewaert and De Vuyst 1996) these parameters were tested as possible methods of improving bacteriocin production by UAL26. Both shaking and stationary cultures showed similar zones of activity (by spot-on-lawn technique) at 18 and 21 hours (Table 6) after the supernatant was concentrated from 1 ml to 250 µl. After 23 hours, no activity was detected. Therefore, aeration was not an effective way of increasing production.

Table 4. Bacteriocin production detected by deferred inhibition from *C. piscicola* UAL26 grown on different media at 25°C in duplicate.*

Growth medium tested **	Activity
Soytone and Tryptone	---
Soytone and Yeast extract	+
Tryptose	---
Casitone and Yeast Extract	+
Casitone and Tryptone	---
Casamino acids and Yeast Extract	+
Casamino acids and Tryptone	---
Yeast Extract and Peptone	+
Yeast Extract and Proteose Peptone	++
Tryptone and Proteose Peptone	---
Yeast Extract (BBL)	+
Yeast Extract (Difco)	++
Tryptone	---
APT	+

*The indicator was *L. monocytogenes* LI0502.
**All media was obtained from Difco except YE from BBL. The base medium was agar.
(---) No activity.
(+) Clear zone of inhibition (<12 mm).
(++) Large zone of inhibition (>12 mm).

Table 5. Effect of varying yeast extract concentration in APT agar on bacteriocin production by *C. piscicola* UAL26.

YE concentration (g/L)	Activity
3.75	---
7.5	+
15	+
30	---
60	---

The indicator was *L. monocytogenes* LI0502.
(---) No activity.
(+) Clear zone of inhibition.

Table 6. Bacteriocin production by *C. piscicola* UAL26 at various growth times, aeration, temperature, and pH.

Parameter	Activity in supernatant*
Shaking at 200 rpm (hours of growth)	
6	--
12	--
14	--
16	--
18	+*
21	+*
23	--
24	--
Stationary (hours of growth)	
6	--
12	--
14	--
16	--
18	+*
21	+*
23	--
24	--
Temperature 25°C	+ (Stationary)
Temperature 30°C	+
Initial pH 6.0	--
Initial pH 7.0	--
Initial pH 8.0	+
pH maintained at 6.0	--
pH maintained at 7.0	--
pH maintained at 8.0	+

* Activity based on spot-on-lawn inhibition
(---) No zone of inhibition.
(+*) Slight zone of clearing
(+) Clear zone of inhibition

Similar activity was detected in cultures grown at 25°C or 30°C for 12 hours and freeze-dried to 25% of the original volume.

UAL26 was grown in APT with an initial pH of 6.0, 7.0, and 8.0. There was an increase in bacteriocin production in APT with starting pH 8.0 (Table 6). Maintaining the pH at 8.0 showed an even greater increase in bacteriocin production, which could be due to the fact that when LAB grow they acidify the medium and inhibit their own growth.

4.4 Optimization of purification of bacteriocin from UAL26.

4.4.1 Optimum recovery of bacteriocin from hydrophobic exchange (XAD-8) and cation exchange (Sepharose) chromatography.

The purpose of hydrophobic exchange chromatography (HEC) was to remove compounds that have different hydrophobic properties from the bacteriocin. The bacteriocin was eluted from the column after successive washing of the XAD-8 column with 30%, 35%, and 40% ethanol (Table 7). Subsequent washing with 50% ethanol did not elute any active compounds. Based on this result a washing protocol was developed for the XAD-8 column, which involved washing the column with 25% ethanol and then eluting the bacteriocin with 40% ethanol.

Identical reasoning (to HEC) was used for the optimization of the SP sepharose cation exchange column. During cation exchange chromatography (CEC) the active component was eluted at 200 mM NaCl. Gradient elution of the bacteriocin from a high performance cation exchange column produced no fractions that had any detectable

7. Recovery of activity during the purification of UAL 26 bacteriocin using heated and unheated supernatant, hydrophobic exchange chromatography, and cation exchange chromatography.

Parameter		Activity
Heated 6 L supernatant (APT and BHI)*		-
Unheated 6 L supernatant (APT and BHI)		-
Hydrophobic exchange (%Ethanol : %Water)		
XAD-8	0%: 100%	-
	20%: 80%	-
	25%: 75%	-
	30%: 70%	+
	35%: 65%	+
	40%: 60%	+
	50%: 50%	-
	60%: 40%	-
Cation exchange (mM NaCl in 20 mM Na acetate)		
Sephadex	0	-
	50	-
	100	-
	150	-
	200	+
	300	-
	400	-
	500	-
	1000	-

*Activity from both the heated and unheated supernatant (grown in BHI or APT) gave similar results.
(-) No activity detected.
(+) Clear zone of inhibition is seen.

activity (results not shown). Another method of improving cation exchange performance was attempted. Peptide was added to the resin at low pH (2.0) and the pH was slowly increased until the peptide disassociated from the column (Williams 1997). This method did not cause release of the bacteriocin (results not shown).

4.4.2 Optimization of the recovery of bacteriocin from HPLC purifications.

4.4.2.1 Isocratic vs gradient separations and the selection of a column ligand.

HPLC purification of the bacteriocin from UAL26 was done using different elution techniques. The data in Table 8 show the retention time of the active component and the amount of activity detected in the most concentrated fraction. Isocratic runs had a shorter retention time than gradient runs. For isocratic elution, previous runs (data not shown) resulted in the bacteriocin eluting from the column with 33% acetonitrile, and 25% 2-propanol. Isocratic elution at 31% acetonitrile did not result in detection of any activity. Isocratic elution is a very common chromatographic method for isolating compounds; however, after more than ten attempts this method of elution gave very little recovery of bacteriocin (a 6 to 7 fold reduction in activity) and was therefore abandoned in HPLC purification of UAL26 bacteriocin.

Various silica-based alkyl packings in HPLC columns are commonly used to separate peptides and proteins. For isolation of the active component from UAL26 C-4, C-8, and C-18 reversed phase HPLC columns were used. Alteration of the column ligand (alkyl chain length) showed a loss of activity with a C-4 (butyl) column from 6400 to 3200 activity units compared with C-8 octyl or C-18 octadecyl columns. There was no

Table 8. Retention time and peak concentration in arbitrary units of high performance liquid chromatography purification of bacteriocin from *C. piscicola* UAL26.

Parameter	**Retention time***	**Peak concentration**
AU/ml		
Isocratic HPLC		
31% Ace N*	-	-
33% Ace N	7 min	100
35% Ace N	6 min	200
40% 2-propanol	4 min	100
Column ligand (alkyl chain length)		
C4 0-80% AceN (0.9%/min)	41-46 min	3200
C8	43-48 min	6400
C18	43-48 min	6400
Temperature effect		
45°C 10-80% AceN (0.9%/min)	20-21 and 23-25 min	400 and 1600
56°C	21-22 and 24-26 min	400 and 1600
65°C	21 and 24-25 min	800 and 3200
pH effect 10-80% AceN (0.9%/min)		
pH 7.0 Na acetate	-	-
Counterion effect		
0.1% phosphoric acid	41-43 min	100
0.1% HFBA**	48-49 min	400
0.05% TFA ***	42-44 min	6400
0.1% TFA	41-43 min	6400
0.5% TFA	28-30 min	1600
Ramping and Solvent effect (230 nm)		
methanol 0.45%/min	135-157 min	400
methanol 0.90%/min	75-91 min	800 and 6400
methanol 1.80%/min	-	-
ethanol 0.45%/min	63-75 min	800 and 12800
ethanol 0.90%/min	57-69 min	12800
2-propanol 0.45%/min	58-70 min	800 and 800
2-propanol 0.9%/min	38-47 min	800 and 6400

*AceN = Acetonitrile
**HFBA = Heptafluorobutyric acid
***TFA = Trifluoroacetic acid

difference in activity (retention time or peak activity) for C-8 or C-18 columns. Recovery of bacteriocin with either a C-8 or C-18 column gave similar results. The C-4 column did not give as much recovery of bacteriocin as the C-8 or C-18 columns (3200 AU versus 6400 AU, respectively). Although the recovery of activity from the C-4 column was within a two-fold dilution of the C-8 and C-18 columns it was omitted from further use because the C-4 column is more susceptible to degradation at low pH (Esser and Unger 1991) which is the pH at which HPLC purifications were performed.

4.4.2.2 Effect of temperature, pH and counterion on bacteriocin resolution.

Samples were passed over an HPLC column at various temperatures to determine if there was an optimal temperature at which the bacteriocin could be eluted (Table 8). Temperature affected the retention time and recovery of bacteriocin. Retention time decreased from one peak at 42 min (room temperature) to two peaks at approximately 21 and 24 min at 45, 55, and 65°C. At 45 and 55°C, recovery of bacteriocin was half of the amount recovered at 65°C, 400 and 1600 versus 800 and 3200 activity units, respectively.

Greater recovery of bacteriocin was obtained when the column was heated to 65°C than 45°C (from 1600 to 3200 AU, respectively). Also, separation improved because recovery occurred over 2 min (at 45, 55, and 65°C) rather than 6 min for ambient temperatures. HPLC chromatography using sodium acetate to adjust the pH to 7 in the mobile phase did not result in the detection of any activity. Although this method of HPLC was attempted once, it was discontinued because there was no recovery of

bacteriocin and HPLC at neutral pH can degrade a column faster than when the column is run at an acidic pH.

Most peptides are charged when in solution and the presence of a counterion will influence their chromatographic behavior (Hodges and Mant 1991). The counterion can bind to the peptide and make the peptide-ion complex more hydrophobic (with heptafluorobutyric acid (HFBA) as the counterion) or hydrophillic (with phosphoric acid as the counterion). Changing the counterion in the solvents resulted in a 6-fold loss in activity when 0.1% phosphoric acid was used and a four-fold loss in activity when 0.1% HFBA was used (Table 8). HFBA is a strongly hydrophobic counterion (Nugent et al. 1988) and as expected it increased retention time. HFBA is not volatile therefore another step would be required to remove it, possibly resulting in the loss of more bacteriocin. It was discontinued from further use. TFA was an excellent counterion because it is completely volatile, minimally detected with UV monitors at low wavelengths (<214 nm) (Hermodson and Mahony 1983), and it was miscible in both organic and inorganic phases. The recovery of bacteriocin was highest when TFA was used compared with HFBA or phosphoric acid. Because TFA proved to be the most effective counterion it was used for all HPLC purifications.

Once TFA was selected as the counterion, changing the concentration of TFA was investigated to improve the resolution of the bacteriocin. Increasing the concentration of the counterion has been shown to alter the retention time of peptides during HPLC (Hodges and Mant 1991). For the bacteriocin produced by UAL26, increasing the TFA concentration to 0.5% shortened the retention time; however, there was a two-fold loss in

activity. Therefore, this method for peptide purification was discontinued. Altering the concentration of TFA from 0.1% to 0.05% did not alter the retention time or the amount of activity recovered. Therefore, 0.05%TFA was chosen as the optimum TFA counterion concentration.

4.4.2.3 Gradient steepness and various organic modifiers

The primary method used for isolating the bacteriocin from UAL26 was gradient elution because isocratic elution gave reduced activity of the samples from the column (see Table 8). Gradient steepness affects the separation of peptides and is often the most effective way to change selectivity (Snyder et al. 1997). Resolution of the bacteriocin from UAL26 improved as the gradient decreased (from 1.80%/min to 0.9%/min). The active peak did not elute at the same percentage of organic solvent because retention time is not the reciprocal of gradient slope (Guo et al. 1986). The highest recovery with the narrowest band-width (active fractions) was found by decreasing the gradient to 0.9%/min, after which, the effect from further decreasing the gradient showed no improvement on recovery. It was found that for each organic modifier an optimum recovery could be associated with a specific gradient (0.9%/min for 2-propanol, 0.45% for ethanol, and 0.9% for methanol). The optimum gradient for resolving the bacteriocin with acetonitrile as an organic modifier was 0.9%/min (results not shown). A higher gradient gave reduced recovery of bacteriocin. Why this occurred is not known. Lowering gradient steepness might be expected to allow for diffusion of the peptide thus reducing recovery and broadening active peaks; however, this phenomenon was not

observed.

The reason for selecting alcohols as organic modifiers in HPLC was to rapidly reduce the volume of crude sample and to isolate as much active peptide as possible. The solvents were chosen for many reasons. The solvents have different hydrophobicities and may cause the bacteriocin to elute at different times from contaminants. From the data in Table 3 it was shown that the active component was stable and soluble in each solvent. Poor solubility of the bacteriocin in the solvent might cause the bacteriocin to precipitate in the column (Hermodson and Mahoney 1983). The solvents were volatile and their later removal was not difficult. Altering the organic phase changed the retention time of the active peptide on the column (see Table 8). With a gradient of 0.45%/min, activity was detected in the fraction collected at 135 min (methanol), 63 min (ethanol), and 58 min (2-propanol). The length of time over which activity was detected, differed from 22 min (methanol) to 12 min (ethanol and 2-propanol). For methanol, increasing the gradient from 0.45%/min to 0.9%/min separated a second peak and increased recovery of activity from 400 AU/ml to 800 and 6400 AU/ml, respectively. Increasing the gradient to 1.8%/min failed to show activity. For ethanol, increasing the gradient from 0.45%/min to 0.9%/min did not show a change in recoverable activity; however, a second peak was observed (800 AU/ml) with the slower gradient. For 2-propanol, increasing the gradient from 0.45%/min to 0.9%/min increased recovery from 800 to 6400 AU/ml.

4.5 Purification of the antibacterial compound from supernatant of UAL26 grown in BHI broth using optimized production and purification conditions.

The data in Tables 7 and 8 show that the optimal fermentation and recovery conditions included: growth in BHI broth at pH 8.0; elution from the XAD-8 column with 40% ethanol after washing the column with 25% ethanol; and elution from the cation exchange column at 200 mM NaCl after washing the column with 150 mM NaCl. The purification of the bacteriocin from BHI was based on the information gained from previous experiments (Tables 7 and 8).

The data in Table 9 illustrate the purification of the second peptide. No activity was detected in the supernatant of *C. piscicola* UAL26 grown in BHI broth. After hydrophobic exchange, activity was detected in the fraction eluted from the column with 40% ethanol. Recovery of active component dropped from 53% to 11% after cation exchange. After cation exchange, NaCl was removed from the sample by Sep pak with 40% acetonitrile. After desalting, recovery was 1%, which represents the greatest loss of active component during the procedure. HPLC with 2-propanol gave 1 ml of solution with 3200 AU/ml and 0.4% recovery. The data in Figure 3 show the elution profile of the peptide isolated after HPLC with ethanol as the organic modifier. It shows one peak at 23 minutes. Figure 4 is a MALDI-TOF MS analysis of the purified peptide showing a single peak at 2596 Da. No other peaks were observed. Electrospray MS also showed a peptide of 2610 Da. (University of Alberta Chemistry Department) (results not shown). A second mass spectral analysis at an independent lab (Department of Pharmaceutical Chemistry in the University of California) showed that the peptide was 2609.485 +/- 0.03 Da. The active sample that was obtained after HPLC with ethanol was not titred. Therefore, the final amount of activity was not known. When mixed with Proteinase K

Table 9. Purification of bacteriocin from *C. piscicola* UAL26 grown in BHI at 25°C. The volume of sample, activity within the sample and % recovery at each step is presented.

Fraction	Volume (L)	Activity AU/ml	% Recovery
Supernatant	10	-	-
Hydrophobic exchange			
XAD-8	3	200	100
Rotovap + freeze dry*	0.02	25,600	85
Size Exclusion			
Sephadex G-50**	0.4	800	53
Cation Exchange			
SP Sepharose	0.04	1600	11
Desalting			
Sep pak	0.005	1600	1
HPLC			
2-propanol	0.001	3200	0.4
Ethanol	0.001	ND	ND

(ND) Not determined.

* During rotovaporization and freeze-drying, precipitate did not dissolve into water.

** 5 ml of sample was added to the size exclusion column. The isolated active component was combined from four runs.

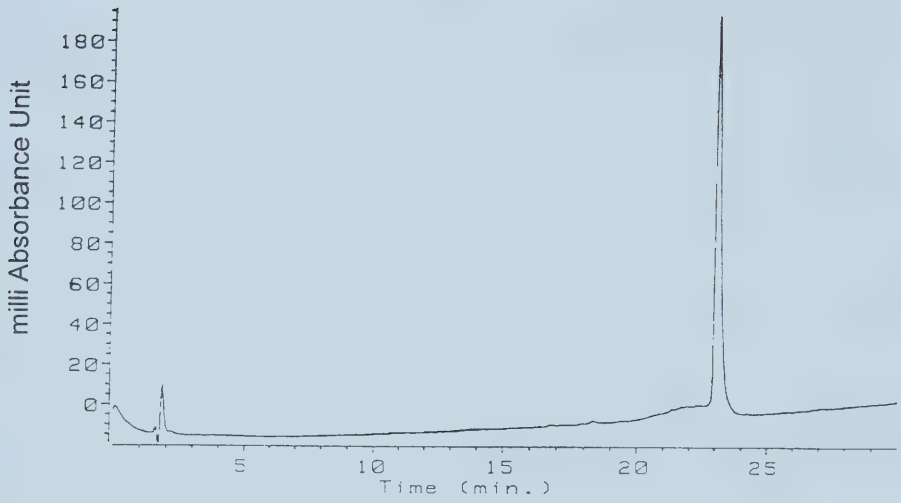

Figure 3.

Elution pattern of the peptide isolated from *C. piscicola* UAL26 grown in BHI broth. The organic modifier was acetonitrile (B) with a flow rate of 0.5 ml/min. The peptide was detected at 212nm. The gradient was 0-80% (B) in 30 minutes.

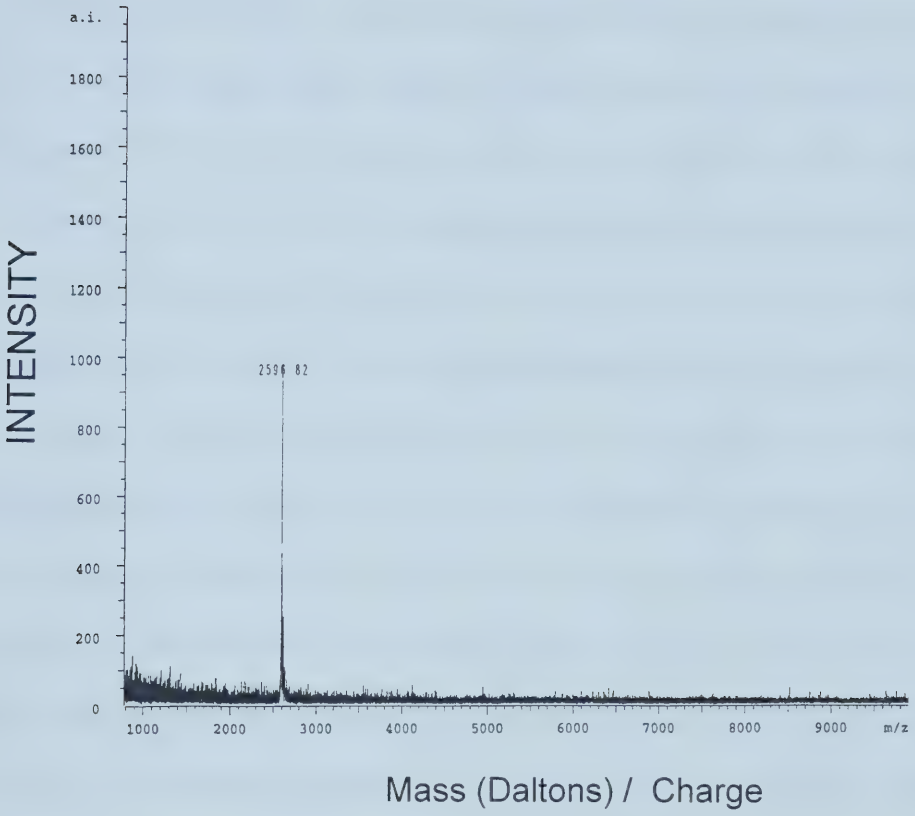

Figure 4.

MALDI-TOF MS analysis of the peptide isolated from the culture supernatant of *C. piscicola* UAL26 grown in BHI broth.

the isolated peptide did not inhibit *L. monocytogenes* LI0502. A two-component bacteriocin was ruled out because inactive fractions that were combined and tested by spot-on-lawn technique did not produce activity against the indicator *L. monocytogenes* LI0502 (results not shown). Because the previous bacteriocin isolation from UAL26 grown in APT broth yielded a peptide from casein, another growth medium was chosen for growth of UAL26. BHI was chosen as the ideal medium because it is a complex medium in which UAL26 grows and produces bacteriocin. BHI was believed not to contain any form of casein. In addition to changing the medium, the production of the bacteriocin was improved by starting the fermentation at pH 8.0. The purification was improved by optimizing hydrophobic exchange (elution at 40% ethanol) and cation exchange (elution at 200 mM NaCl). Recovery from the final purification step (HPLC) was optimized by heating (to 65°C) a C-18 reversed phase column with 2-propanol and ethanol as organic modifiers (see Table 9). After the isolation of the second peptide, a second DNA probe was synthesized. This probe did not anneal to the template genomic DNA. Upon further investigation, it was revealed that Difco adds casein to BHI to bring the medium to performance specifications.

4.6 Synthesis of the second peptide.

Given the purity of the peptide, as determined by Electrospray MS and MALDI-TOF MS (Figure 4), and the fact that a previously isolated antibacterial peptide had been characterized from casein (Zucht et al. 1995), it was assumed that the active component was the casein peptide. To determine if the isolated peptide was the antibacterial

compound, it was synthesized.

The data in Table 10 indicate the sequence of the three synthesized peptides. The first peptide was synthesized because it contained sequences from both α and β-casein. αs_1 casein amino acids 1 to 6 were linked to β-casein amino acids 69 to 92 (Kunji et al. 1996). From Edman degradation analysis, there was one type of peptide being sequenced.

The second peptide was the primary peptide of interest. From Figure 3 (HPLC analysis), Figure 4 MALDI-TOF MS analysis, and Mass Spectral analysis (performed in the Department of Chemistry at the University of Alberta and at the Department of Pharmaceutical Chemistry at the University of California) it was believed to be pure. The isolated peptide also produced a clear zone of inhibition against an indicator lawn of *L. monocytogenes* LI0502. This peptide was synthesized to determine if the amino acid sequence was the active component from UAL26 grown in a medium containing casein.

The third peptide was synthesized because the overlapping sequence of the first two peptides was identical (Table 10). Previous samples that were purified (but not sequenced) did not show the same sized peptides in all of the active fractions. If this core region was the reason for inhibition, it would explain why active fractions from UAL26 supernatant often produced different sized peptides, i.e., the core region was responsible for inhibition and N-terminally or C-terminally linked amino acids accounted for differences in active peptide size.

Peptides 1 and 2 were sequenced by Edman degradation (Table 10). The complete peptide could not be sequenced due to deteriorating signal. From the molecular weight of

Table 10. Amino acid sequence of peptides obtained from the American Peptide Company and peptides sequenced during the purification of the active component from UAL26.

		Mol. Weight (Da.)
Peptide 1		
Sequence	NH_2 - RPKHPINIPPLTQTPVVVPPFLQP - COOH	2686
Synthesized	NH_2 - RPKHPINIPPLTQTPVVVPPFLQPEV - COOH	2892
Peptide 2		
Sequence	NH_2 - SLPQNIPPLTQTPVVVPPFLQPE - COOH	2510
Synthesized	NH_2 - SLPQNIPPLTQTPVVVPPFLQPEV - COOH	2609
Peptide 3		
Synthesized	NH_2 - NIPPLTQTPVVVPPFLQPEV - COOH	2255

*For peptides that were sequenced, the entire sequence of the peptide could not be determined by Edman degradation. The peptides were synthesized based on the sequence obtained from Edman Degradation and the final amino acids were predicted from Electrospray MS.

the purified peptide (estimated by MALDI-TOF MS for peptide 1 and by electrospray for peptide 2), the final carboxyl-terminal amino acid for both peptides was estimated. The first two peptides were then synthesized. The third peptide was synthesized to be identical to the overlapping regions of the first two peptides and called the 'core' region.

No activity was detected for any of the samples tested at a concentration of 1 mg/ml or 50 mg/ml (in the case of peptide 2). Later purification using tissue culture medium (MEM, RPMI, and Williams E), which contains no foreign protein, showed activity against *L. monocytogenes* LI0502 if the medium was concentrated 50 times (results not shown). Therefore, based on these two observations, the sequences from peptides 1, 2, and 3 are probably not the active components found in the culture supernatant produced by UAL26.

5. Discussion

5.1 Purification of the first peptide isolated from UAL26 and probing the genome of UAL26.

The purpose of the initial purification was to isolate a sufficient quantity of peptide that would swamp any contaminating peptides during amino acid sequencing by Edman degradation (Matsudaira 1993). The supernatant was heated to inactivate any proteases present (Law and Kolstad 1983). The hydrophobic exchange step is a well-characterized method for taking advantage of the hydrophobicity of the peptide to separate it from peptides with different hydrophobicities. It is a rapid method for reducing the volume of the sample. The theory behind cation exchange chromatography was to selectively isolate the peptide of interest based on the charge of the peptide.

C. piscicola UAL26 is a fastidious organism. The selection of a growth medium for bacteriocin production by UAL26 is critical for its isolation. To test for maximum bacteriocin production, the organism was grown in complex media (APT and BHI broth) and tissue culture media (MEM, RPMI and Williams E). Bacteriocin production was ten-times greater in the complex media than in the tissue culture media (results not shown). Therefore, for production purposes, complex media were more efficient. However, BHI and APT contain several contaminating peptides in the range of 3,000 to 6,000 Da. (Carolissen-Mackay et al. 1996). The presence of peptide contaminants in the media make downstream processing more difficult. With UAL26 this was confirmed by the isolation of peptides (casein hydrolysates) that were eluted with the active component after hydrophobic exchange, cation exchange, and HPLC. Attempts at supplementing

minimal media to increase bacteriocin production may therefore be a more promising method of isolating the bacteriocin.

The loss in recovery of bacteriocin during the purification of the peptide represents a significant obstacle to its purification. A 50% loss in activity during purification is not uncommon (Pohl 1990). However, as reported in Tables 2 and 9, the final recovery of bacteriocin was 0.5% and 0.4% of the initial amount that was detected. The loss of activity of bacteriocins has been reported to be related to the purity of the bacteriocin in solution meaning that, as the bacteriocin becomes more and more pure it becomes inactivated (Hastings et al. 1991; Worobo et al. 1994). However, pure solutions of synthesized bacteriocin such as leucocin A have been found to be stable (Fregreau-Gallagher et al. 1997). Another possibility for the loss of activity is adsorption of the bacteriocin to the side of test tubes during the purification (Carolissen-Mackay et al. 1996).

In this study, reverse genetics was to be attempted to isolate the bacteriocin gene. Reverse genetics involves sequencing part of a peptide produced by the gene and then locating the bacteriocin gene based on this code. The specificity of the probe sequence that was synthesized based on the first peptide that was isolated was increased in three ways. Because lactic acid bacteria have a higher AT to GC nucleotide base ratio (Pot et al. 1994), where a choice between any of the four bases had to be made, A and T were selected over G and C. Second, the third base in a codon sequence has the most instability, i.e., whether it is A, T, G, or C will produce the same amino acid. Therefore, the probe sequence ended with the first two bases of the final codon. Third, data banks of

previously identified and sequenced *Carnobacterium piscicola* genes provided probabilities of codons for amino acids in that species.

In the current study, the probe did not bind to a complementary sequence on the chromosome. There are many possibilities for this. First, if there was a sequencing error the probe from which the peptide sequence was derived would not be complementary to the gene. Second, if the synthesized oligomer was too short and/or contained too many unstable codons, it may anneal randomly to the genome. Under less stringent conditions, nonspecific binding can occur. Third, if probe labeling was not efficient, the annealed probe would not be detected. Fourth, if the peptide isolated did not originate from the organism, there may not be a complementary sequence on the target genome. Later comparisons of the peptide sequence with Peptools Biotools Inc., (Edmonton, AB.) database showed that the sequence from which the DNA probe was synthesized was of bovine origin. The peptide sequence had 100% complementarity with a portion of bovine β-casein (NIPPLTQTPVVVPPFLQPEV) and α-casein (RPKHPI). Although it is possible for two different species to contain similar genes, this possibility was considered unlikely because it was determined that the medium (APT broth) contained peptides derived from bovine casein.

Several LAB are known to produce enzymes (proteases) capable of cleaving peptides at specific sites in the amino acid sequence (Kunji et al. 1996). These enzymes have been shown to be associated with the cell wall, intracellularly, and exported into the extracellular environment. From this study it was shown that two peptides from β-casein were isolated from the culture supernatant of C. *piscicola* UAL26 grown in APT broth

and BHI broth. Although it is possible that these peptides originated from the media, it is also possible that *C. piscicola* UAL26 produced a protease capable of cleaving β-casein yielding the peptide that was isolated (see Table 9). Among the known LAB capable of cleaving β-casein, *Lactococccus lactis* H2 cleaves β-casein at similar sites to the peptide isolated in this study. Coolbear et al. (1992) showed that *L. lactis* H2 is capable of cleaving bovine β-casein between positions 68 and 93 yielding a 25 amino acid peptide (SLPQNIPPLTQTPVVVPPFLQPEVM). The peptide isolated from the present study yielded a 24 amino acid peptide from position 68 to 92 of bovine β-casein (SLPQNIPPLTQTPVVVPPFLQPEV). Should UAL26 produce a protease which specifically recognizes and cleaves the amino acid residue between valine and methionine at postion 92 it may be a novel protease.

5.2 *Optimizing the production of bacteriocin from UAL26.*

Before a second attempt at purifying a bacteriocin from UAL26 was made, methods for optimizing production were investigated to aid in the purification. Optimizing bacteriocin production can result in greater yields and simplify downstream purification because recovery of bacteriocin from crude extract can result in 'dramatic losses' during purification (Muriana and Luchansky 1993). The peptide does not have to be absolutely pure to be sequenced. If the molarity of the desired peptide is greater than the contaminants in the solution, the peptide may be sequencable. A 1 to 10 pmol amount of pure peptide is required for sequencing 20 to 40 amino acids (Matsudaira 1993). To sequence subsequent amino acids, up to 100 pmol of peptide is required.

5.3 Optimizing the purification of bacteriocin from UAL26.

Once the parameters necessary for optimizing the production of the bacteriocin were determined, methods to improve the purification scheme were investigated to maximize recovery of the bacteriocin. The main technique used for purification was HPLC. There are many methods for separating peptides on HPLC. These include variations in temperature, counterion, pH, flow rate, organic modifier, gradient, column ligand, and column length (Mant and Hodges 1991). HPLC was chosen as the final purification step to purify the bacteriocin of UAL26 because the use of HPLC has been successful in purifying several bacteriocins (Carolissen-Mackay et al. 1996). HPLC was optimized because the partitioning window (range over which the organic modifier can be adjusted to separate a peptide from its nearest impurity) is very narrow for peptides (Hodges and Mant 1991). The loss in activity from the chromatography steps may be due to the fact that only active fractions were pooled. Fractions that contained bacteriocin below the detection level of spot-on-lawn assay technique were discarded.

5.4 Isolation of the second peptide.

In the current study, detection of the antibacterial compound produced by UAL26 involved MALDI-TOF MS, electrospray MS, and U.V. (Ultraviolet light) detection. Because some peptides do not ionize efficiently in MALDI-TOF MS or electrospray MS they do not provide a good indication of peptide purity (Andreas et al. 1997; Metzger et al. 1994). U.V. is a good indicator of peptide purity (Metzger et al. 1994; Settineri and Burlingame 1996). U.V. analysis (Figure 3) showed a single symmetrical peak at 23

minutes therefore, no contaminating peptides were detected. Given the fact that the compound that was isolated from BHI broth showed a single peak under U.V. analysis and was inactivated by proteinase K, it was believed to be the purified proteinaceous compound in the fully-grown culture supernatant of UAL26.

Casein is a constituent of APT broth and may be found in BHI broth (Difco). Because activity was detected when UAL26 was grown in a medium containing hydrolyzed casein (BHI and APT), it was postulated that a cleavage product of β-casein could be the component responsible for the activity against *L. monocytogenes* LI0502. Once the peptides were isolated and shown to be active, they were sequenced and synthesized. The synthesized peptide was not active against *L. monocytogenes* LI0502 or *C. piscicola* UAL9 therefore; the proteinaceous antibacterial compound produced by UAL26 was not isolated. If a portion of casein is not the active peptide, it is likely that the bacteriocin is highly active and below the detection limit of MALDI-TOF MS and electrospray (below nanomolar concentrations) with similar hydrophobic and ionic properties to the casein peptide isolated.

References

Ahn, C., and M.E. Stiles. 1990. Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. J. Appl. Bacteriol. 69: 302-310.

Ahn, C., and M.E. Stiles. 1992. Mobilization and expression of bacteriocin plasmids from *Carnobacterium piscicola* isolated from meat. J. Appl. Bacteriol. 73: 217-228.

Andreas, F., R. Huhmer, G.I. Aced, M.D. Perkins, R.N. Gursoy, D.S.S. Jois, C. Larive, T.J. Siahaan, and C. Schoneich. 1997. Separation and analysis of peptides and proteins. Anal. Chem. 69: 29R-57R.

Aymerich, T., H.Holo, L.S. Havarstein, M. Hugas, M. Garriga, and I.F. Nes. 1996. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium,* a new antilisterial bacteriocin in the pediocin family of bacteriocins. Appl. Environ. Microbiol. 62: 1676-1682.

Benoit, V., A. Lebrishi, J.B. Millier, and G. Lefebvre. 1997. Purification and partial amino acid sequence of brevicin 27, a bacteriocin produced by *Lactobacillus brevis* SB27. Curr. Microbiol. 34: 173-179.

Bhunia, A.K., Johnson, M.C., and B. Ray. 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici.* J. Appl.

Bacteriol. 65: 261-268.

Blom, H., T. Katla, A. Holck, K. Sletten, L. Axelsson, and H. Holo. 1999. Characterization, production, and purification of leucocin H, a two-peptide bacteriocin from *Leuconostoc* MF215B. Curr. Microbiol. 39: 43-48.

Bogovič-Matjašić, B., Rogelj, I. F. Nes, and H. Holo. 1998. Isolation and characterization of two bacteriocins of *Lactobacillus acidophilus* LF221. Appl. Microbiol. Biotechnol. 49: 606-612.

Burns, K.A. 1987. Potential of a lactic acid bacterium for the biological preservation of ground beef. MSc. thesis, University of Alberta.

Callewaert, R. and L. De Vuyst. 1996. Purification and improved production of amylovorin L471, a bacteriocin from *Lactobacillus amylovorus* DCE 471. Med. Fac. Landbouw. Univ. Gent. 61: 1565-1572.

Carolissen-Mackay, V., G. Arends, and J.W. Hastings. 1996. Purification of bacteriocins of lactic acid bacteria: problems and pointers. Int J. Food Microbiol. 34: 1-16.

Casaus, P., T. Nilsen, L.M. Cintas, I.F. Nes, P.E. Hernandez, and H. Holo. 1997. Enterocin B, a new bacteriocin from *Enterococcus faecium* BFE T136 which can act

synergistically with enterocin A. Microbiology 143: 2287-2294.

Chopin, A. 1993. Organization and regulation of genes for amino acid biosynthesis in lactic acid bacteria. FEMS Microbiol. Rev. 12: 21-38.

Cintas, L.M., P. Casuas, L.S. Håvarstein, P.E. Hernandez, and I.F. Nes. 1997. Biochemical and genetic characterization of enterocin P, a novel *sec*-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. 63: 4321-4330.

Cintas, L.M., P. Casuas, H. Holo, P.E. Hernandez, I.F. Nes, and L.S. Havarstein. 1998. Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50, are related to staphylococcal hemolysins. J. Bacteriol. 180: 1988-1994.

Cintas, L.M., Rodriguez, J.M., M.F. Fernandez, K. Sletten, I.F. Nes, P.E. Hernandez and H. Holo. 1995. Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad inhibitory spectrum. Appl. Environ. Microbiol. 61: 2643-2648.

Collins, M.D., J.A.E. Farrow, B.A. Phillips, S. Ferusu, and D. Jones. 1987. Classification of *Lactobacillus divergens*, *Lactobacillus piscicola* and some catalase negative, asporogenous, rod shaped bacteria from poultry in a new genus, *Carnobacterium.* Int. J.

Syst. Bacteriol. 37: 310-316.

Coolbear, T., J.R. Read, and G.G. Pritchard. 1992. Stability and specificity of the cell-wall associated proteinase from *Lactococcus lactis* subsp. *cremoris* H2 released by treatment with lysozyme in the presence of calcium ions. Appl. Environ. Microbiol. 58: 3263-3270.

Coventry, M.J., J. Wan, R.F. Gordon, R.F. Mawson, and M.W. Hickey. 1996. Production of brevicin 286 VB286 and partial characterization. J. Appl. Microbiol. 80: 91-98.

De Vuyst, L. and E.J. Vandamme. 1994. Lactic acid bacteria and bacteriocins: their practical importance. In *Bacteriocins of Lactic Acid Bacteria,* ed. De Vuyst, L., and E.J. Vandamme. New York: Blackie Academic and Professional, pp. 1-12.

Esser, U. and K.K. Unger. 1991. Reversed phase packings for the separation of peptides and proteins by means of gradient elution high performance liquid chromatography. In *High Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis, and Conformation,* ed. Mant, C.T. and Hodges R.S. Florida, CRC Press Inc., pp. 273-278.

Fiat, A., D. Migliore-Samour, and P. Jolles. 1993. Biologically active peptides from milk

proteins with emphasis on two examples concerning anti-thrombotic and immunomodulating activities. J. Dairy Sci. 76: 301-310.

Franz, C.M.A.P. 1999. PhD thesis. Department of Agriculture Food and Nutritional Sciences at the University of Alberta.

Franz, C.M.A.P., U. Schillinger, and W.H. Holzapfel. 1996. Production and characterization of enterocin 900, a bacteriocin produced by *Enterococcus faecium* BFE 900 from black olives. Int. J. Food Microbiol. 29: 255-270.

Franz, C.M.A.P., R.W. Worobo, L.E.N. Quadri, U. Schillinger, W.H. Holzapfel, J.C. Vederas, and M.E. Stiles. 1999. Atypical genetic locus associated with constitutive production of enterocin B by *Enterococcus faecium* BFE 900. Appl. Environ. Microbiol. 65: 2170-2178.

Fregreau-Gallagher, N.L., M. Sailer, W.P. Niemczura, T.T. Nakashima, M.E. Stiles, and J.C. Vederas. 1997. Three-dimensional structure of leucocin A in trifluoroethanol and dodecylphosphocholine micelles: spacial location of residues critical for biological activityin type IIa bacteriocins from lactic acid bacteria. Biochemistry. 36: 15062-15072.

Galvez, A., G. Gimenez-Gallego, M. Maqueda, and E. Valdivia. 1989. Purification and amino acid composition of peptide antibiotic AS-48 produced by *Streptococcus*

(Enterococcus) faecalis subsp. *liquefaciens* S-48. Antimicrob. Agents Chemother. 33: 437-441.

Galvez, A., M. Maqueda, E. Valdiva, A. Quesada, and E. Montoya. 1986. Characterization and partial purification of a broad spectrum antibiotic AS-48 produced by *Streptococcus faecalis*. Can J. Microbiol. 32: 765-771.

Gao, Y. 1998. Unpublished work. Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton.

Gao, Y., M.J. van Belkum, and M.E. Stiles. 1999. The outer membrane of gram-negative bacteria inhibits antibacterial activity of brochocin-C. Appl. Environ. Microbiol. 65: 4329-4333.

Gonzalez, C.F. and B.S. Kunka. 1987. Plasmid associated bacteriocin production and sucrose fermentation in *Pediococcus acidilactici.* Appl. Environ. Microbiol. 53: 2534-2538.

Gratia, A. 1925. Sur un remarquable exemple d'antagonisme entre souches de colibacille. C.R. Soc. Biol. 93: 1040-1042.

Guo, D., C.T. Mant, A.K. Taneja, and R.S. Hodges. 1986. Prediction of peptide retention

times in reversed phase high performance liquid chromatography. II. Correlation of observed and predicted peptide retention times and factors influencing the retention times of peptides. J. Chromatogr. 359: 519-532.

Hanlin, M.B., N. Kalchayanand, P. Ray, B. Ray. 1993. Bacteriocins of lactic acid bacteria in combination have greater antibacterial activity. J. Food Prot. 56: 252-255.

Hastings, J.W., M. Sailer, K. Johnson, K. L. Roy, J.C. Vederas, and M.E. Stiles. 1991. Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum.* J. Bacteriol. 173: 7491-7500.

Hermodson, M., and W.C. Mahony. 1983. Separation of peptides by reversed phase high performance liquid chromatography. Meth. Enzymol. 91: 352-359.

Hodges, R.S., and C.T. Mant. 1991. Properties of peptides/proteins and practical implications. In *High Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis, and Conformation*, ed. Mant, C.T. and R.S. Hodges. Florida, CRC Press Inc., pp. 3-11.

Hsieh H., and B.A. Glatz. 1996. Long term storage stability of the bacteriocin propionicin PLG-1 produced by *Propionibacterium thoenii* and potential as a food preservative. J.

Food Prot. 59: 481-486.

Irvine, G.B. 1997. Size exclusion high performance liquid chromatography of peptides. Anal. Chim. Acta. 352: 387-397.

Ivanova, I., V. Miteva, T. Stefanova, A. Pantev, I. Budakov, S. Danova, P. Moncheva, I. Nikolova, X. Dousset, and P. Boyaval. 1998. Characterization of a bacteriocin produced by *Streptococcus thermophilus* 81. Int. J. Food Microbiol. 42: 147-158.

Josic, D. and W. Reutter. 1991. Sample preparation and application of different HPLC methods for the separation of hydrophobic membrane proteins. In *High Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis, and Conformation,* ed. Mant, C.T., and R.S. Hodges. Florida, CRC Press Inc., pp. 231-238.

Kanatani, K., M. Oshimura, K. Sano. 1995. Isolation and characterization of acidocin A and cloning of the bacteriocin gene from *Lactobacillus acidophilus.* Appl. Environ. Microbiol. 61: 1061-1067.

Katchalsky, K., E. Lahov, and M.T. Sode-Morgensen. 1973. Polypeptidic antibiotic substances derived from casein. U.S. Patent 3, 764, 670.

Klaenhammer, T.R. 1993. Genetics of bacteriocins produced by lactic acid bacteria.

FEMS Microbiol. Rev. 12: 39-86.

Kunji, E.R.S., I. Mierau, A. Hagting, B. Poolman, and W.N. Konings. 1996. The proteolytic systems of lactic acid bacteria. Antonie van Leeuwenhoek 70: 187-221.

Lahov, E. 1972. Unpublished. Refer to Lahov and Regelson 1996.

Lahov, E., and W. Regelson. 1996. Antibacterial and immunostimulating casein-derived substances from milk: casecidin, isracidin peptides. Food Chem. Toxicol. 34: 131-145.

Laukova, A., M. Marekova, and P. Javorsky. 1993. Detection and antimicrobial spectrum of a bacteriocin like substance produced by *Enterococcus faecium* CCM4231. Lett. Appl. Microbiol. 16: 257-260.

Law, B.A., and J. Kolstad. 1983. Proteolytic systems in lactic acid bacteria. Antonie van Leeuwenhoek 49: 225-245.

Lozano, J.C.N., J.N. Meyer, K. Sletten, K. Pelaz, and I.F. Nes. 1992. Purification and amino acid sequence of a bacteriocin produced by *Pediococcus acidilactici.* J. Gen. Microbiol. 138: 1985-1990.

Lyon, W.J., and B.A. Glatz. 1991. Partial purification and characterization of a

bacteriocin produced by *Propionibacterium thoenii.* Appl. Environ. Microbiol. 57: 701-706.

Lyon, W.J., and B.A. Glatz. 1993. Isolation and purification of propionicin PLG-1, a bacteriocin produced by a strain of *Propionibacterium thoenii.* Appl. Environ. Microbiol. 59: 83-88.

Lyon, W.J., J.K. Sethi, and B.A. Glatz. 1993. Inhibition of psychrotrophic organisms by propionicin PLG-1, a bacteriocin produced by *Propionibacterium thoenii.* J. Dairy Sci. 76: 1506-1513.

Mant, C.T., and R.S. Hodges. 1991. The effects of anionic ion pairing reagents on peptide retention in reversed phase chromatography. In *High Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis, and Conformation,* ed. Mant, C.T. and R.S. Hodges. Florida: CRC Press Inc., pp. 327-342.

Marciset, O., M.C. Jeronimus-Stratingh, B. Mollet, and B. Poolman. 1997. Thermophilin 13, a nontypical antilisterial poration complex bacteriocin, that functions without a receptor. J. Biol. Chem. 272: 14277-14284.

Martinez-Bueno, M., M. Maqueda, A. Galvez, B. Samyn, J. van Beeumen, J. Coyette, and E. Valdivia. 1994. Determination of the gene sequence and the molecular structure of

the enterococcal peptide antibiotic AS-48. Appl. Environ. Microbiol. 176: 6334-6339.

Martinez-Bueno, M., E. Valdivia, A. Galvez, J. Coyette, and M. Maqueda. 1998. Analysis of the gene cluster involved in production and immunity of the peptide antibiotic AS-48 in *Enterococcus faecalis.* Mol. Microbiol. 27: 347-358.

Matsudaira, P. 1993. Introduction. In *A Practical Guide to Protein and Peptide Purification for Microsequencing*, ed. Matsudaira, P. San Diego: Academic Press Inc., pp. 3-12.

McCormick, J.K., A. Poon, M. Sailer, Y. Gao, K.L. Roy, L.M. McMullen, J.C. Vederas, M.E. Stiles, M.J. van Belkum. 1998. Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. Appl. Environ. Microbiol. 64: 4757-4766.

Mendoza, F., M. Maqueda, A. Galvez, M. Martinez-Bueno, and E. Valdiva. 1999. Antilisterial activity of peptide AS-48 and study of changes induced in the cell envelope properties of an AS-48 adapted strain of *L. monocytogenes.* Appl. Environ. Microbiol. 65: 618-625.

Metzger, J.W., K. Christoph, K. Wiesmuller, and G. Jung. 1994. Electrospray mass spectrometry and tandem mass spectrometry of synthetic multicomponent peptide

mixrtures: determination of composition and purity. Anal. Biochem. 219: 261-277.

Miller, M.J.S., S.A. Witherly, and D.A. Clark. 1990. Casein: a milk protein with diverse biologic consequences. Proc. Soc. Exp. Biol. Med. 195: 143-159.

Motlagh, A.M., A.K. Bhunia, F. Szostek, T. Hansen, M.G. Johnson, and B. Ray. 1992. Nucleotide and amino acid sequence of pap-gene (pediocin AcH production) in *Pediococcus acidilactici* H. Lett. Appl. Microbiol. 15: 45-48.

Muriana, P.M., and J.B. Luchansky. 1993. Biochemical methods for purification of bacteriocins. In *Bacteriocins of Lactic Acid Bacteria,* ed. Hoover D.G. and Steenson, L.R. San Diego: Academic Press Inc., pp. 44-57.

Nes, I.F., D.B. Diep, L.S. Håvarstein, M.B. Brurberg, V. Eijsink, and H. Holo. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie van Leeuwenhoek 70: 113-128.

Nes, I.F. and H. Holo. 2000. Class II antimicrobial peptides from lactic acid bacteria. Biopoly. 55:50-61.

Nissen-Meyer, J., and I.F. Nes. 1997. Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action. Arch. Microbiol. 167: 67-

77.

Nugent, K.D., W.G. Burton, T.K. Slattery, B.F. Johnson, and L.R. Snyder. 1988. Separation of proteins by high performance liquid chromatography. II. Optimizing sample pretreatment and mobile phase conditions. J. Chromatogr. 443: 381-397.

O'Keeffe, T., C. Hill, and R.P. Ross. 1999. Characterization and heterologous expression of the genes encoding enterocin A production, immunity, and regulation in *Enterococcus faecium* DPC1146. Appl. Environ. Microbiol. 65: 1506-1515.

Pohl, T. 1990. Concentration of proteins and removal of solutes. Meth. Enzymol. 182: 68-89.

Pot, B, W. Ludwig, K. Kersters, and K. Schleifer. 1994. Taxonomy of lactic acid bacteria. In *Bacteriocins of lactic acid bacteria,* ed. De Vuyst, L., and E.J. Vandamme. New York: Blackie Academic and Professional, pp. 14-67.

Quadri, L.E.N., M. Sailer, K.L. Roy, J.C. Vederas, and M.E. Stiles. 1994. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV 17B. J. Biol. Chem. 269: 12204-12211.

Reiter, B., and G. Harnulv. 1984. Lactoperoxidase antibacterial system: natural

occurrence, biological functions and practical applications. J. Food Prot. 47: 724-742.

Rekhif, N., A. Atrih, and G. Lefebvre. 1994. Selection and properties of spontaneous mutants of *Listeria monocytogenes.* Curr. Microbiol. 28: 237-241.

Rogers, L.A. 1928. The inhibiting effect of *Streptococcus lactis* on *Lactobacillus bulgaricus.* J. Bacteriol. 16: 321.

Rose, N.L., P. Sporns, and L. McMullen. 1999a. Detection of bacteriocins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Appl. Environ. Microbiol. 65: 2238-2242.

Rose, N.L., P. Sporns, M.E. Stiles, and L.M. McMullen. 1999b. Inactivation of glutathione in fresh meat. J. Food Sci. 64: 759-762.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Settineri, C.A., and A.L. Burlingame. 1996. Structural characterization of protein glycosylation using HPLC/electrospray ionization mass spectrometry and glycosidase digestion. In *Protein and peptide analysis,* ed. J.R. Chapman. Totowa; New Jersey: Humana Press Inc., pp. 255-278.

Shaw B.G., and C.D. Harding. 1984. A numerical taxonomic study of lactic acid bacteria from vacuum packaged beef, pork, lamb and bacon. J. Appl. Bacteriol. 56: 25-40.

Siragusa, G.R. and C.N. Cutter. 1993. Brochocin-C, a new bacteriocin produced by *Brochothrix campestris.* Appl. Environ. Microbiol. 59: 2326-2328.

Snyder, L.R., J.J. Kirkland, and J.L. Glajch. 1997. Practical HPLC Method Development, 2nd ed. New York: John Wiley & Sons Inc., pp. 351-400.

Stiles, M.E. 1994. Bacteriocins produced by *Carnobacterium* species. In *Bacteriocins of Lactic Acid Bacteria*, ed. L. de Vuyst and E.J. Vandamme. London; New York: Blackie Academic and Professional, pp. 497-506.

Stiles, M.E. 1996. Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek 70: 331-345.

Suma, K., M.C. Misra, M.C. and Varadaraj. 1998. Plantaricin LP84, a broad spectrum heat-stable bacteriocin of *Lactobacillus plantarum* NCIM 2084 produced in a simple glucose broth medium. Int. J. Food Microbiol. 40: 17-25.

Tagg, J.R., A.S. Dajani, and L.W. Wannamaker. 1976. Bacteriocins of gram-positive

bacteria. Bacteriol. Rev. 40: 722-756.

van Belkum, M.J., and M.E. Stiles. 1995. Molecular characterization of genes involved in the production of the bacteriocin leucocin A from *Leuconostoc gelidum.* Appl. Environ. Microbiol. 61: 3573-3579.

van Belkum, M.J., and M.E. Stiles. 2000. Nonlantibiotic antibacterial peptides from lactic acid bacteria. Nat. Prod. Rep. 17: 323-335.

Wade, L.G. 1987. Amino acids, peptides and proteins. In *Organic Chemistry,* ed. L.D. Wade. New Jersey, Prentice Hall Inc., pp. 1214-1263.

Whittal, R. 1999. Personnal communication. Department of Chemistry, University of Alberta.

Williams, A. 1997. Pharmacia Biotechnology. Information from Amersham Pharmacia Biotechnology. University of Alberta.

Worobo, R.W., T. Henkel, M. Sailer, K.L. Roy, J.C. Vederas, and M.E. Stiles, 1994. Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A. Microbiology 140: 517-526.

Worobo, R.W., M.J. van Belkum, M. Sailer, K.L. Roy, J.C. Vederas, and M.E. Stiles. 1995. A signal peptide secretion dependent bacteriocin from *Carnobacterium divergens*. Appl. Environ. Microbiol: 3143-3149.

Zucht, H.D., M. Raida, K. Adermann, H.J. Magert, and W.G. Forssmann. 1995. Casocidin-I: a casein- αs_2 derived peptide exhibits antibacterial activity. FEBS Lett. 372: 185-188.